A STUDY OF THE EARLY DEVELOPMENT OF TOOTH GERMS

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Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Science.
February, 1970.

CONTENTS

Page No.

SUMMARY

Organ culture techniques have been used to find out at what stage in development the embryonic mouse mandible becomes determined for tooth formation, and what parts the epithelial and mesenchymal components play in determining tooth morphology.

The normal stages of tooth development in the strain of mouse used in later culture experiments were first examined, special attention being paid to the development of morphological features, and to the distribution of collagen fibrils, glycogen, neutral and acid mucopolysaccharides. This series acted as a control against which tooth development was measured under the experimental conditions.

Mandibular arches from successively younger ages of embryo of 14-8 days gestation age were isolated and grown in organ culture. The ability to self-differentiate into tooth germs in culture was regarded as the expression of determination of tissues for tooth formation. It was found that:

a) Mandibles of 10-14 day old embryos are determined for the formation of tooth germs with specific incisor or molar morphology. Calcification of the dentine, and the deposition of enamel matrix are missing, and it is possible that determination for those activities is lacking at the time of explantation.

b) Mandibular arches of 9-day, and late 8-day old embryos, seem determined for the formation of tooth germs with the morphology of the late cap stage, but seem undetermined for the formation of the specific pattern of cell division responsible for cusp morphology.

c) Mandibular arches of 8-day old embryos seem undetermined for tooth formation.

Mandibular/

Mandibular odontogenic epithelial and mesenchymal tissues were separated by trypsin-pancreatin and cultured either as the isolated layers, or after recombination.

Isolated epithelium and mesenchyme survived in culture, but showed no differentiation to odontogenic tissues.

The majority of the separated tissues were recombined, either as in vivo, or with incisor epithelium combined with molar mesenchyme and vice versa. Culture of such recombinations gave the following results:

a) Mandibles from 8-day embryos, although viable in culture after such treatment, seemed incapable of forming epidermal derivatives.

b) Mandibles from 9- and 10-day embryos were incapable of survival under the present conditions.

c) Mandibles from 11- and 12-day embryos were capable of forming tooth germs in culture.

The morphology of these tooth germs, and the position of their development within the recombined explants, indicate that:

i) specific mesenchyme may be responsible for inducing and maintaining tooth formation.

ii) mesenchyme appears to determine the axis of development of the incisor within the mandible.

iii) epithelium, at the 12-day, and possibly at the 11-day stage (i.e. at the stage of early dental lamina formation), is responsible for the development of the specific incisor or molar pattern of the future tooth germ.

PREFACE

This thesis describes an investigation of tissue reactions involved in the development of the dentition of the mouse.

One of the most remarkable features of a dentition is the regularity with which the teeth develop in characteristic number and form in any one species. How such an orderly arrangement arises is a problem which has attracted palaeodontologist, geneticist and embryologist alike.

To the palaeodontologist, the teeth of mammals are thought to preserve all the essential features of the placoid scale of the Elasmobranch. Each is a hard structure piercing the epidermis, with a denticle consisting of a pulp cavity enclosed by dentine and surmounted by an enamel cap. Their attachments too, are similar. The placoid scale has a basal plate of cement, the mammalian tooth has a root portion of dentine covered by cementum. In both, the pulp, dentine and cement are mesodermal derivatives, butthe enamel has an epithelial origin.

Many theories attempt to trace the evolution of the multicusped tooth of the mammalian heterodont dentition from such a simple conical tooth form. Chief of these are the tritubercular theory of Osborn (1923) and the Dimer theory of Bolk (1921, 1922). Osborn believes that the component cusps of the complex teeth of the higher mammals may be identified with the triangle, or trigon, of cusps found in a considerable number of early mammals. Bolk believes that mammalian tooth form is the result of fusion of the anlagen of two reptilian tooth germs, and cites the presence of localisations of condensed cells in the developing embryonic enamel organ as an indication of its dual origin. A wholly satisfactory theory, however, has not yet been proposed. The tritubercular theory is confined to the origin of premolars and molars and does not/

not consider how the tritubercular tooth of the early mammal arose. On the other hand, palaeontological evidence for the Dimer theory is lacking, and its acceptance would demand more substantiation from fossil sources.

The geneticist has had more success in his search for mechanisms controlling tooth morphology. What evidence there is, of the genetic control of the dentition of the mouse, has been gleaned by Grüneberg (1951, 1965, 1966). In a survey of over forty mouse mutants, he could find only three genes which affected the dentition as a whole. He found that the crown pattern of the molars, and the size of the mandibular incisors and molars were each dependent on the activity of these genes. The development of the second and third molars seemed to be dependent on the size of the first molar, as enlargement of it in some mutants caused a corresponding decrease in the size of the other two, and vice versa. Grüneberg interpreted these findings as suggesting that in normal development the first lower molar competitively inhibits the growth of the later formed molars.

Thus it can be seen that at the multi-cellular level the expression of the activity of the genes controlling tooth development can apparantly be modified by physiological control. It is at this tissue level that investigations of tooth development by embryologists have been carried out.

It had long been postulated that tooth formation must conform to the normal embryonic mechanisms of organogenesis where one tissue which is 'competent' reacts and directs its future development under the 'inducing' or 'evocating' action of another. Experimental proof, however, had to wait until certain experimental methods had been devised.

The technique of tissue culture was devised in the early 1900's by Ross Harrison (1907). It was simple and uniquely suitable for assessing the specific effects of one group of cells on/

on another. Any participation in normal development by migrating cells, nerve-borne stimuli, hormone or other blood-borne stimuli from developing tissues elsewhere in the body, were automatically excluded with the isolation of the tissues in question from the rest of the body. The culture conditions did, however, supply all 'normal' maintenance factors such as plasma, humidity, correct temperature, to allow the tissues to continue in their already 'programmed' path of development.

In the 1930's, Glasstone introduced this method to an investigation of tooth development, and brought to a more precise level investigations of developmental mechanisms previously less accurately carried out by transplantation techniques. Her earliest results (1936, 1938a, 1938b) showed that once the tooth germ was in the cap stage showing early cusp formation, not only was its future pattern of cusp development independent of general non-mandibular stimuli, but it was also independent of the influence of the surrounding cells within the mandible.

Attention was now concentrated on this small volume of tissue - the isolated tooth germ. The way that epithelial and mesenchymal elements developed side by side in an intricate pattern of great constancy led observers to the belief that the epithelium and mesenchyme were developmentally interdependent. Indeed, the interdependence of these components of the tooth germ had been tested by mechanical separation of the two layers, followed by transplantation of the parts into living hosts. Tissue culture techniques now afforded a more refined method of investigation, where all possible interference of inducing actions of the host were excluded. Lefkowitz and his colleagues (1944, 1947), Niizima (1956), Koch (1965), Pourtois (1966) and others attempted to solve the problem. Their experiments revealed many fascinating properties of the odontogenic cells, but except for the most recent experiments, failed to prove interdependence between the epithelial and mesenchymal components, partly because the delicate mechanical dissections of the earlier experiments could not allow a complete separation of the two layers.

In/

In 1952 the Mosconas published their results of experiments where cells had been separated from each other without damage, by enzymatic (trypsin) action. Further experiments (Grobstein & Auerbach, 1958) led to a method of separating the tissue layers cleanly at the basement membrane, leaving intact undamaged tissues. Pourtois (1966) and Koch (1967) made use of the enzymatic technique to separate the tooth germ into its mesenchymal and epithelial components, and contributed substantially to our knowledge of interactions between the two layers. Both workers found that tooth germs separated in this way and recombined, continued to develop normally in culture, forming dentine and enamel matrix. Both found that the enamel organ failed to differentiate in isolation, and Koch found that collagen gel, known to support differentiation of some isolated epithelia (Dodson, 1967) failed to support it in the tooth. Koch could find no sign of odontoblast differentiation when the dental papilla was cultured in isolation, but Pourtois suggested that on cytochemical and morphological evidence preodontoblasts could be identified with difficulty. Dentine matrix was not formed. Thus it could be seen that there was strong evidence for an interdependence of the tissue layers.

Koch (1967) carried our knowledge a stage further. The epithelial and mesenchymal layers, when recombined on either side of a Millipore filter and cultured, differentiated to form their relevant matrices as though the filter had replaced the basement membrane. Cell to cell contact between the layers was unlikely through the pores, though the possibility should not be overlooked. Ameloblasts were never observed in the absence of odontoblasts on the opposite filter face, and differentiation of odontoblasts was undoubtedly related to interaction with the internal layer of the enamel organ. Moreover, there was clear proof that materials were deposited into the filter by the odontoblasts and ameloblasts, indicating that substances may be passed to the other layer under <u>in vivo</u> conditions.

The/

The experiments quoted above, all describe investigations of the induction phenomena involved in tooth germ formation, after the cap stage has been reached. Few experiments have been reported in the literature describing interactions at earlier stages. Hay (1956, 1961), Fisher (1957) and Pourtois (1964) confirmed Glasstone's results on the self-differentiation of tissues at the cap stage, and they found by isolating successively less mature mandibles, that the tissues were self-differentiating as soon as the first morphological signs of tooth formation could be seen. Hay (1961) found that in one isolated case, this could be true even earlier.

In this work, I have therefore confined my attention to investigating the induction phenomena associated with the earliest stages of tooth development. The experiments I am going to describe were designed to find out at what stage in development the mandibular tissues of the mouse become biologically 'determined' for tooth formation, and to investigate interactions taking place between the odontogenic epithelium and mesenchyme. The work is reported here in three parts:-

Part 1 - describes the day-by-day morphological, histological and histochemical development of the mandible in the particular strain of mouse used in the experimental work. This part is essentially the control series for the following two parts, i.e. it shows normal *in vivo* development.

Part 11 - describes experimental work, where mandibles of the mouse are isolated from successively younger ages, and cultured *in vitro* to find out at what stage the tissues are independent of external developmental influences and are self-differentiating, i.e. 'determined' for tooth formation. This series shows normal *in vitro* tooth development, and therefore forms a further control series for Part 111 of this work.

Part/

Part III - describes experiments where the epithelium and mesenchyme of the mandible are separated by enzymatic action, recombined in various ways and cultured *in vitro*, to study the epigenetics, or causal interactions between the various parts of the developing teeth.

PART 1.

IN VIVO DEVELOPMENT OF THE MANDIBULAR ARCH OF THE MOUSE.

INTRODUCTION

In Part 1 of the thesis, tooth development in the mouse will be considered first of all in general terms, then more specifically as it is found in the strain of mouse used by me for experimental purposes.

Several papers have been published correlating gestation age with specific features of mouse mandible formation. The earlier workers tended to mention few stages, and confined themselves only to certain aspects of development. Mahn (1890) described the order of development and eruption of mouse teeth, ignoring much of the morphodifferentiation, and the histodifferentiation of the cusps and ridges; Hoffmann (1925) concentrated his attention on incisor tooth development and on a few selected aspects, such as the enamel knot, of molar tooth development, and Bhaskar, Weinmann and Schour (1948) correlated certain radiological and histological appearances of the mandible with age, without giving morphological details.

Gaunt (1955) gave the first detailed description of the morphological development of the molar tooth germ of the mouse from 10-21 days gestation age, and in 1956 he extended his observations to the formation of dentine and enamel with special reference to the enamel free areas. Hay (1956) and Fisher (1957) included in their Ph.D theses, detailed descriptions of the histological development of mouse mandibles. Hay (1956) was concerned with incisor, cartilage and bone formation from 9-18 days gestation, and Fisher (1957) with molar development from 13 days in utero to 2 days after birth.

These morphological and histological investigations were extended by Milaire (1959) and Pourtois (1961) to include the biochemical changes in the tissues, as revealed by histochemical methods/

methods. Milaire (1959) turned his attention to histochemical appearances in the cephalic regions of the mouse embryo of 8-11 days gestation, while Pourtois (1961) confined his to the appearances of the tooth germ from 10-21 days. He later (1962) examined similar changes during the deposition of the hard tissues.

Throughout the thesis, brief mention will be made of some of the other derivatives of the first branchial arch, as they make their appearance in the results of the experimental work, and are of interest in comparison with tooth development. Meckel's cartilage was described during its early development by Milaire (1959) and in its later stages by Hay (1953, 1956). Hay (1956) also described developmental changes in the dentary. Borghèse (1950a) described in detail the histodifferentiation of the submandibular and major sublingual glands. The thyroid gland is of passing interest, as a derivative arising between the first and second branchial arches. There are no accounts (except in Japanese and so far unobtainable), of normal early thyroid development in the mouse.

Detailed as these descriptions of mouse tooth development are, they are clearly unsuitable for use as standards of normal for later experimental work in this study. There is general agreement about the sequence of events in the morphological and histological development of the mandible, but their timing is more controversial. The first signs of odontogenic activity - the epithelial thickening in the mid-line axis of the arches - was first seen before the 10 day stage by Gaunt (1955), early on the 10th day by Milaire (1959), at 11 days by Pourtois (1961) and at 12 days by Cohn (1957). Subsequent development is also at variance.

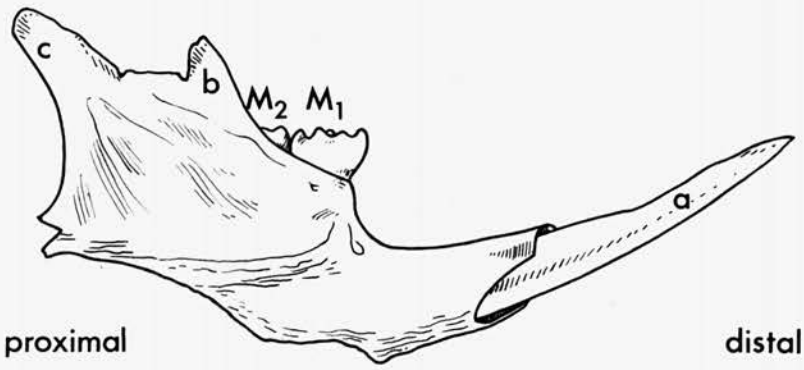
Where the descriptions did closely correspond with mine, a wide enough range of ages was not included to be useful as standards of/

of comparison in my experimental work. Milaire's work (1959) is the only description which begins early enough (8 days) for my purposes, but does not proceed beyond 11 days. Pourtois' descriptions (1961) extend to an old enough stage, but begin at 10 days in utero, two days too late.

However, by compounding their results and the sporadic references to mouse embryonic tooth development given by other workers while describing experimental work on the mouse dentition, a sequence of events can be found which enables identification of the various parts of the developing tooth, and gives an idea of the sequence of events.

For clarity in the rest of the thesis, a resumé of normal tooth development based on these reports, is given here to introduce the various terms met with later.

LATERAL VIEW OF RIGHT MOUSE MANDIBLE

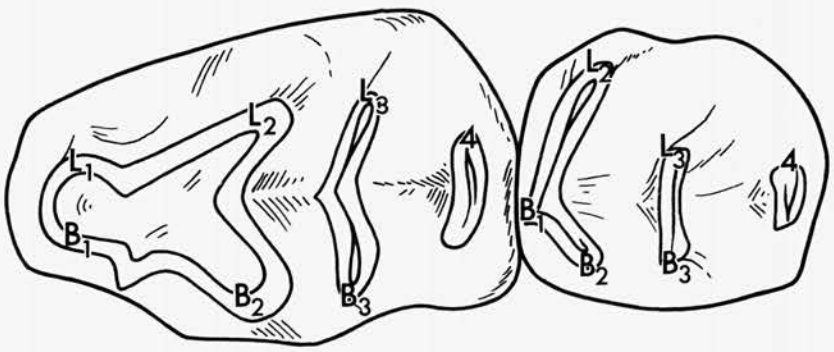


a – incisor
b – coronoid process
c – condylar process

Fig. I.

1st & 2nd LEFT MANDIBULAR MOLAR TEETH

EZL strain of Mouse — occlusal view



M_1	M_2
cusps present	cusps present
$L_1 B_1$: $L_2 B_2$: $L_3 B_3$: cusp 4	B_1: $L_2 B_2$: $L_3 B_3$: cusp 4

Fig. 2.

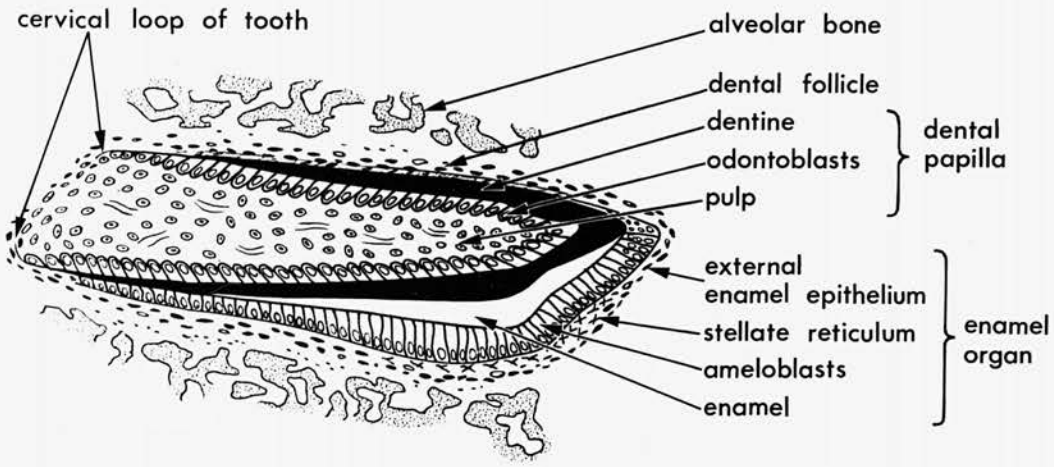


DIAGRAM OF INCISOR AT BELL STAGE OF DEVELOPMENT

Fig. 3.

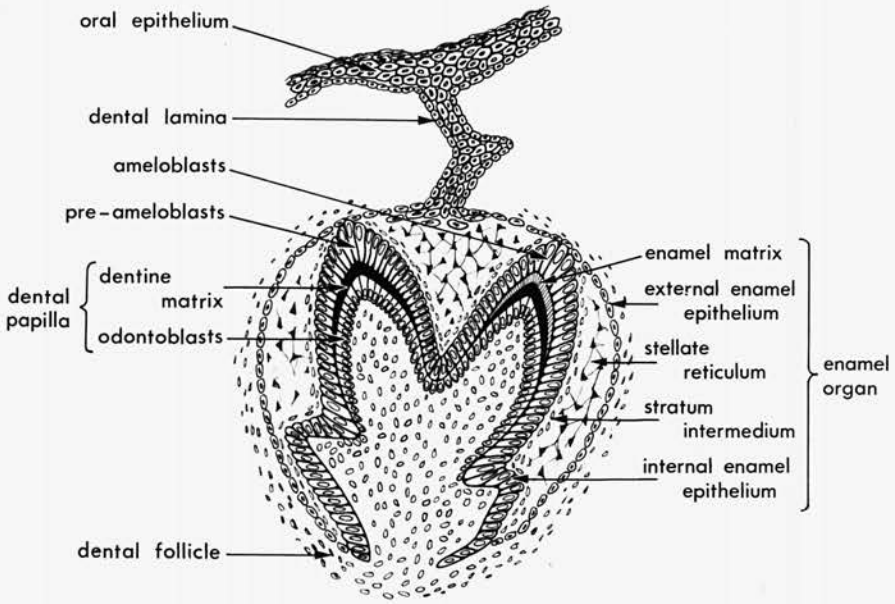


DIAGRAM OF MOLAR AT BELL STAGE OF DEVELOPMENT

Fig. 4.

General description of the development of the mouse mandibular dentition.

a) Morphological Development.

The dental formula of the monophyodont dentition of the mouse is I $\frac{1}{1}$: C $\frac{0}{0}$: PM $\frac{0}{0}$: M $\frac{3}{3}$, i.e. 16 teeth in all. The incisors are of persistent growth, and are spatulate in form. Enamel covers the convex labial surface, leaving only the tip uncovered, and the lingual surface of the underlying dentine covered by cementum. The molar teeth are of limited growth, and in the lower jaw, the crowns are composed of two rows of cusps connected by lophs (ridges) and separated by sulci (clefts). The dentine of the crown is covered with enamel except for the anterior slopes of the tips of the cusps and the lophs, which are enamel-free.(Gaunt, 1956). Following the terminology of Gaunt (1955), the lower molars from distal to proximal in the jaw are M_1 : M_2 : M_3. The lingual cusps are similarly L_1 : L_2 : L_3 and the buccal cusps B_1 : B_2 : B_3 (Figs. 1 & 2). M_1 is composed of cusps L_1 B_1 : L_2 B_2 : L_3 B_3 and a small posterior unpaired cusp, cusp 4. M_2 is composed of cusps L_2 B_2 : L_3 B_3 : 4 according to Gaunt, but Grüneberg (1965) found that in most strains of mouse which he examined, B_1 was present. Grüneberg suggests that B_1 was missing in Gaunt's strain as a mutation in the inbred strain used. M_3 forms after birth, and is of no immediate interest in this study.

The histological changes that occur during ontogeny of the teeth, are far from simple. These changes (together with some mention of those of other derivatives of the mandibular arch), will be described in detail.

b) Histological Development.

The mandibular, or first branchial arches, arise as two separate/

separate lateral swellings cephalad to the developing heart. The ectoderm is 2-3 cells thick, and overlies undifferentiated mesenchyme (Hay, 1956; Milaire, 1959; Pourtois, 1961). The blood supply is formed by the first arch of the aorta, but shortly after its establishment in the arches, it degenerates and is replaced by uniformly distributed capillaries (Milaire, 1959; Pourtois, 1961).

The arches grow in volume and fuse in the mid-line. The first signs of tooth development are seen once the capillaries are formed (Gulat, 1936; Milaire, 1959; Pourtois, 1961). Three separate in-growths of epithelium into the mesenchyme are seen (Hay, 1956, 1961). The lip furrow band and the two incisor tooth germs arise from the anterior ingrowth, and two posterior ingrowths - one in each arch - give rise to the molar tooth germs. In some strains of mouse, the ingrowth of epithelium - or dental lamina as it is called - is continuous throughout the length of the jaw (Hoffmann, 1925; Pourtois, 1961).

i) Incisor tooth germ.

The anterior epithelial invagination forms two distinct parts early in its formation (Hay, 1961). The outer, anterior part forms the lip furrow band which later opens to form the buccal sulcus separating the teeth from the lips. The inner, lingual part forms the incisor tooth germs. It becomes, on each side, a club shaped invagination, surrounded by a dense condensation of deeply staining mesenchymal cells (Milaire, 1959; Pourtois, 1961; Hay, 1961). The blood supply is dense, particularly between it and the lip furrow band (Milaire, 1959; Pourtois, 1961; Hay, 1961). The deep surface of the epithelial bud becomes hollowed out, the peripheral cells dividing in such a way that a cap, and then a bell shape of epithelium is formed, enclosing the condensed mesenchyme (Fig. 3). The lip of the bell on the labial surface grows proximally more extensively than the lingual side, rotating the tooth germ so that the open face of the bell is turned proximally/

proximally (dorsally). The epithelial bell is now called the enamel organ and the mesenchyme which it encloses, is the dental papilla (Fig. 3). The mesenchyme immediately adjacent to the outside of the bell is also condensed. It is called the dental follicle and later forms the supporting structure of the tooth.

The enamel organ soon differentiates into three layers. The outer layer is composed of cuboidal cells and is called the external enamel epithelium. The cells of the inner layer, aligned against the basement membrane (membrana praeformativa; Butler, 1956), on the inside of the bell, are slightly taller and form the internal enamel epithelium. The line of reflection of the basement membrane, where the external enamel epithelium and the internal enamel epithelium meet, is called the cervical loop. Originally the cells lying between the external and internal enamel epithelia are relatively undifferentiated, and similar to the stratum spinosum of the oral epithelium, but gradually they form two tissues, particularly distinct on the labial side where the enamel organ is thickest and where enamel is going to be formed. A layer of flattened cells, about two cells thick, lies next to the internal enamel epithelium. This is the stratum intermedium. A central mass of stellate-shaped cells surrounded by an increasing volume of intercellular fluid is called the stellate reticulum. The enamel organ, with these differentiating layers, elongates proximally in the jaws until it comes to lie beneath the first, then second and eventually the third molar.

Successive waves of differentiation leading to the formation of hard tissue begin at the anterior labial tip of the tooth germ. The most anterior cells of the internal enamel epithelium elongate to a columnar shape, with centrally placed nuclei. A wave of differentiation passes proximally and lingually down the tooth germ. The elongated cells are called pre-ameloblasts. Shortly after the first pre-ameloblasts have formed changes can be seen in the cells of the dental papilla adjacent to the basement membrane supporting the pre-ameloblasts, and successive changes in this layer pass proximally down the tooth germ, lagging slightly behind/

behind those of the internal enamel epithelium. The peripheral cells of the dental papilla elongate and attach themselves to the basement membrane underlying the pre-ameloblasts. They are called pre-odontoblasts, but are shortly converted into functional odontoblasts with the formation by them of dentine matrix. The first sign of hard tissue formation is the appearance of collagen fibrils between the odontoblasts, fanning out towards the basement membrane (Nylen & Scott, 1958). Mantle predentine, containing as a high proportion of its content these collagen - or Korff's fibres - is the first matrix to appear and is only a few microns in thickness. Circumpulpal predentine is then formed, with a lesser collagen content. The most fully differentiated cells produce predentine first, the wave of matrix formation paralleling the wave of cellular differentiation. Now, lagging behind the wave of predentine formation, pre-enamel is formed on the opposite side of the basement membrane by the now functional ameloblasts on the labial surface of the tooth germ. Whereas the odontoblasts leave cytoplasmic extensions within the predentine as they retreat from the basement membrane, the ameloblasts secrete enamel matrix precursors as granules, and fibrillogenesis occurs extracellularly (Nuckolls, Saunders & Frisbie, 1943; Suga, 1959; Fearnhead, 1960; Nylen & Scott, 1958; Watson, 1960; Reith, 1961). Some disagreement as to actual matrix formation by the ameloblasts is found amongst these workers, but in general it is agreed that the precursors are excreted by the cells - the cell membrane remaining intact. The enamel matrix is prismatic in nature, the central prisms lying at right angles to the basement membrane, now called the amelo-dentinal junction, the ones formed later, more peripherally, are laid down in a herringbone arrangement (Mellanby, 1939; Fleming & Greenfield, 1954; Nylen & Scott, 1958; Pourtois, 1962; Helmcke & Rau, 1962).

Maturation of both dentine and enamel matrix occurs first at the basement membrane spreading peripherally as did the matrix formation/

formation, then calcification may be seen in the matrix - staining blue with haematoxylin (Mellanby, 1939; Rubach & Van Huysen, 1961; Gavin, 1965). Dentine at the tip of the tooth, not covered by enamel, is irregular or osteodentine, containing no tubules (Tomes, 1923; Glasstone, 1936; Mellanby, 1939; Hay, 1961).

The other tissues in the enamel organ and dental papilla have not been quiescent during this period. The most noticeable changes have been in the enamel organ. Blood capillaires have come to lie in furrows in the external enamel epithelium, in juxtaposition to the pre-ameloblast layer, and the external enamel epithelium has degenerated to some extent (Gulat, 1936; Glasstone, 1954; 1962; Hay, 1961; Adams, 1962). Addison & Appleton (1915), Glasstone (1954a) and Pannese (1961), have all emphasised that the formation of enamel matrix is closely linked with vascularisation of the enamel organ.

The changes in the dental papilla, or pulp, are not so dramatic. The blood vessels found are of greater calibre than in earlier stages, and the collagen fibres, especially near the odontoblasts, are coarser.

After eruption, the incisor teeth are kept in occlusion by proliferation of the proximal cells of the enamel organ and pulp compensating for the loss of tooth tissue by attrition at the incisal tip (Chiba, 1965; Hwang & Tonna, 1966).

ii) Molar tooth germ.

The molar tooth germ follows the same course of development as the incisor, except that whereas the incisor elongates proximally in the jaw, the molar tooth germ develops with the open face of the bell-shaped enamel organ facing the inferior surface of the mandible. The morphology of molar teeth, with cusps, lophs and sulci, demands a complicated folding of the basement membrane separating the internal enamel epithelium from the dental papilla, and involves the maintenance of the shape during the deposition of enamel/

enamel and dentine. As with the incisor, the enamel organ of the molar is composed of external enamel epithelium, stellate reticulum, stratum intermedium and internal enamel epithelium (Fig. 4). The tips of the cusps - the future enamel free areas - result from the local cessation of proliferation of the cells of the internal enamel epithelium in these regions (Blechschmidt, 1953; Butler & Ramadan, 1962; Ramadan & Sadek, 1962). The foldings of the basement membrane to form the walls of the crown and the sulci are formed by bands of intense mitotic activity in the internal enamel epithelium (Butler & Ramadan, 1962). The final morphology of the crown of the tooth closely parallels the outline of the basement membrane, except for uneven thicknesses of enamel laid down by the ameloblasts (Glasstone, 1939; Kraus, Kitamura & Latham, 1966).

Both enamel and dentine matrices are laid down against the basement membrane after sequences of cellular differentiation of the internal enamel epithelium and papilla, as in the incisor. The first changes are seen in cusps L_2 B_2 in M_1, at the tips of the cusps, spreading laterally and basally to L_1 B_1 and L_3 B_3, and finally to cusp 4. Similar changes are seen in M_2.

Waves of maturation, as in the incisor, spread from the basement membrane to the peripheries of the newly formed hard tissues, and changes in the enamel organ and dental papilla are similar to those of the incisor. The changes in the enamel organ are very striking. Extravascular erythrocytes and blood vessels appear to penetrate into the stellate reticulum in abundance, with degeneration of the external enamel epithelium (Glasstone, 1954a, 1962; Pannese, 1961). More recently, Decker (1967), in an electron microscopic study of the vascular supply to the rat molar enamel organ, showed that the external enamel epithelial surface continuity was not broken, but that the capillary endothelium and the enamel organ remained separated from each other by their respective basal laminae.

c)/

c) Histochemical Development.

i) Early development.

Although routine histological staining methods show no morphological signs of differentiation of the epithelial and mesenchymal cells in the very young mandible, specific histochemical techniques reveal that cytochemical differentiation is already taking place. Milaire (1959) demonstrated an enlarging halo of cells rich in cytoplasmic glycogen around the first arch of the aorta which supplies the mandibular arches. The cells nearest the vessels were noted to show more glycogen than those more remotely situated. Cells staining intensely for the enzyme alkaline phosphatase have a similar distribution.

The first arch of the aorta degenerates within a few hours, and is replaced by a mass of capillaries. Its original course is marked by the region of cells containing alkaline phosphatase and now indicates the primordium of Meckel's cartilage (Milaire, 1959; Pourtois, 1961). At this stage, the mandibular mesenchyme can be seen to be sharply demarcated into two regions - the central region containing a high alkaline phosphatase content, and an area caphaled to this, extending to the stomodeal ectoderm which is composed of closely packed cells, very rich in cytoplasmic ribonucleic acid (R.N.A.). The stomodeal ectoderm overlying this region is composed of cells rich in both R.N.A. and in slowly increasing quantities of alkaline phosphatase.

The dental laminae appear with little disturbance of this histochemical picture. Similarly to the oral ectoderm, the ingrowths contain a high R.N.A. and alkaline phosphatase content (found also in the pig, Johnson & Bevelander, 1954). The subjacent mesenchymal cells also remain rich in R.N.A. (Milaire, 1959). Pourtois (1961) believes that there is an absence of the densely staining cells in the diastema between the future incisor and molar regions, although a dense vascular capillary bed is uniformly/

uniformly distributed throughout the region.

Alkaline phosphatase begins to disappear at this time - the primordium of Meckel's cartilage being the first to show the decrease. Alkaline phosphatase is lost except for the distal region where cells are differentiating and forming the distal extension of the blastema. The maturing proximal cells gradually acquire glycogen.

The stomodeal ectoderm loses its alkaline phosphatase more gradually, and its cells also begin to accumulate glycogen. Milaire's (1959) and Pourtois' (1961) findings on these points are confirmed by Chiquoine (1957). Glycogen also appears, this time without the alkaline phosphatase precursor, in the mesenchymal cells, not immediately adjacent to the dental lamina, but forming a 'shell' around the future tooth bearing regions.

It is at this stage that Meckel's cartilage becomes morphologically distinct. Its histochemical characteristics are the complete absence of the alkaline phosphatase that first distinguished it, and the high concentrations of intracellular R.N.A. and glycogen. The first tooth germs also show morpho-differentiation now, as localised thickenings on the dental lamina. Glycogen granules become increasingly abundant in the superficial layers of the oral epithelium and the dental lamina, and in the mesenchymal shell around the developing tooth germs.

The first signs of cytodifferentiation of the dentary are seen now as a localised band of cells, external and cephalad to Meckel's cartilage, containing high concentrations of alkaline phosphatase.

The thickenings on the dental lamina develop until the cap stage of development of the molar and incisor tooth germs are seen.

ii) Cap stage of development of tooth germs.

We are again indebted to Pourtois (1961, 1962) for the most detailed descriptions of the histochemical development of the teeth from/

from the cap to the bell stage. Glasstone (1958) described similar stages in the mouse embryo, but studied only the distribution of glycogen. For comparison of their results with others of a similar nature, we must turn to less detailed reports on the development of the tooth germ of the rat - for example those of Horowitz (1942), Saunders, Nuckolls & Frisbie (1942, 1943), Engel (1948), Greep, Fisher & Morse (1948), Sasso & Castro (1957), Suga (1959), Klingsberg, Cancellaro & Butcher (1961), Scheinmann, Weinreb & Wolman (1962), Baratieri (1964) and Porter & Lefkowitz (1965). The guinea pig was studied by Creighton (1896) and the guinea pig and hamster by Suga (1959). The hamster and rat were included in the work already mentioned, by Glasstone in 1958.

The works of Hess & Lee (1952), Ten Cate (1956; 1957; 1962; 1967) and McFall & Kraus (1963), Wislocki, Singer & Waldo (1948) and Wislocki & Sognnaes (1950) on human tooth development are of passing interest, as is that of Bevelander & Johnson (1946; 1955) on pig tooth development.

Many discrepancies are to be found in the reported histochemical state of supposedly comparable developmental stages. They stem from differences in the methods of fixation and staining, from unspecified stages of development or from comparison of results from dissimilar species. Horowitz (1942), for example, found that developing tongue papillae in the human embryo contained glycogen and no alkaline phosphatase, but rat tongue papillae at a comparable stage of development contained alkaline phosphatase and no glycogen.

At the cap stage of development of the tooth germ of the mouse, Pourtois (1961) found that the dental lamina contains intracellular glycogen granules, and continues to do so during the period covered by his description. Pourtois' finding is in agreement with those of Horowitz (1942), Engel (1948), Wislocki, Singer & Waldo (1948), Dalq (1953), Sheinmann, Weinreb & Wolman (1962)/

(1962), McFall & Kraus (1963) and Porter & Lefkowitz (1965). The oral ectoderm also contains glycogen (Sundberg, 1924; Engel, 1948; Wislocki, Singer & Waldo, 1948). Wislocki & Sognnaes (1950) and Dalq (1953) emphasise that it is the superficial cell layers that contain the polysaccharide granules.

The enamel organ of the molar tooth germ was found by Pourtois (1961) at the cap stage, to contain no glycogen, but the shell of mesenchymal cells surrounding the enamel organ and dental papilla acquires increasing quantities. Glasstone (1958), on the other hand, observed the granules in the central mass of enamel organ cells at the cap stage, and in the stellate reticulum as soon as it differentiates. The basement membrane due to its glycoprotein content (Gersh & Catchpole, 1949) is, and remains, P.A.S. positive. The cells in the regions of proximal proliferation of the internal enamel epithelium contain the most densely staining cytoplasmic R.N.A. (Pourtois, 1961). In the molar it is in the lingual wall of the internal enamel epithelium, whereas in the incisor it is in the labial wall. These same cells lose any alkaline phosphatase they contain. Alkaline phosphatase does, however, remain in the developing stellate reticulum.

iii) Bell stage of development of tooth germs.

Molar tooth germ.

External enamel epithelium. At the bell stage of development of the molar tooth germ, Pourtois (1961) found that glycogen granules are present in the cells of the external enamel epithelium. This is in agreement with Creighton (1896), Sundberg (1924), Wislocki et al. (1948), Wislocki & Sognnaes (1950) and Ten Cate (1957). Pourtois found it in most abundance in the buccal wall - significantly the buccal wall does not elongate as rapidly as the lingual side at this stage. The lingual wall, on the other hand, contains the cells in the internal enamel epithelium which stain most intensely/

intensely for R.N.A.. Alkaline phosphatase also increases in these cells.

Stellate reticulum. It is agreed by most workers that glycogen is present in the cells of the stellate reticulum (Creighton, 1896; Sundberg, 1924; Santoné, 1935; Glock, 1940; Engel, 1948; Wislocki, Singer & Waldo, 1948; Wislocki & Sognnaes, 1950; Bevelander & Johnson, 1955; Ten Cate, 1957, and Glasstone, 1958). Horowitz (1942) appears to be the only worker to disagree with this finding.

As the intercellular material increases, and the mucopolysaccharide content rises there (Pourtois, 1961), so the glycogen in the cells disappears (Glock, 1940). At the late bell stage, acid mucopolysaccharide accumulates intercellularly (Bevelander & Johnson, 1955; Butler, 1956; Pourtois, 1961; Baratieri, 1964).

From the beginning of the bell stage, alkaline phosphatase is gradually lost from the stellate reticulum (Pourtois, 1961).

Stratum intermedium. At the same time as the alkaline phosphatase is lost from the cells of the stellate reticulum, it accumulates rapidly in the cells of the stratum intermedium until, at the late bell stage, it is their most distinguishing feature. (Horowitz, 1942; Greep, Fisher & Morse, 1948; Dalq & Mulnard, 1953; Avery, 1954a, and Pourtois, 1961). Santoné (1935) states that the stratum intermedium entirely lacks glycogen.

Internal enamel epithelium. The internal enamel epithelium at first contains but little glycogen (Glock, 1940; Horowitz, 1942; Bevelander & Johnson, 1946). Sundberg (1924) disagrees and is of the opinion that it contains none at all. However, just before deposition of the predentine by the odontoblasts, the preameloblasts that have differentiated from the internal enamel epithelium at the tips of the cusps, become rich in glycogen (Bevelander/

(Bevelander & Johnson, 1946; Glasstone, 1958; Pourtois, 1961). Engel (1948) and Bevelander & Johnson (1955) stress that the glycogen granules are confined to the infra-nuclear zone of the pre-ameloblast.

The R.N.A. content of the cells of the internal enamel epithelium, differentiating into pre-ameloblasts, increase in intensity of staining (Wislocki & Sognnaes, 1950; Dalq, 1953; Pourtois, 1961).

In general, the cells of the internal enamel epithelium are found to contain no alkaline phosphatase except for the cells in the cervical loop region(Bevelander & Johnson, 1945; Pourtois, 1961). Morse & Greep (1952) disagree, and state that no alkaline phosphatase is present anywhere in the internal enamel epithelium.

Dental papilla. The presence of glycogen granules in the developing dental papilla of the molar tooth germ has been confirmed by Wislocki, Singer & Waldo (1948), Wislocki & Sognnaes (1950) and Russell (1967). Engel (1948) and Glasstone (1958) found it to be present in the molar dental papilla only occasionally. Glock (1940), Horowitz (1942) and Bevelander & Johnson (1946) found no glycogen in the dental papilla. Russell (1967) found that the amount of glycogen increased in the bovine dental papilla during development, in the central zones.

Neutral mucopolysaccharides have a similar central distribution (Wislocki & Sognnaes, 1950; Cederberg, 1951; Bevelander & Johnson, 1955; Pourtois, 1961). Wislocki, Singer & Waldo (1948), however, find it distributed intercellularly most abundantly at the periphery of the pulp.

Acid mucopolysaccharides, according to Wislocki, Singer & Waldo (1948), Sasso & Castro (1957), Pourtois (1961), have a similar distribution to the neutral mucopolysaccharides.

Pourtois/

Pourtois (1961) found little alkaline phosphatase in the cuspal region of the dental papilla - a fact disputed by Greep, Fisher & Morse (1948) who found the highest concentrations in the dental papilla in those regions. As the pre-odontoblasts differentiated, these peripheral cells were found to have a high concentration of cytoplasmic R.N.A. (Pourtois, 1961; Ten Cate, 1967).

Incisor tooth germ.

According to Pourtois (1961), at the cap stage of development, the external enamel epithelial cells containing glycogen are those of the labial wall. The mesenchymal cells surrounding the tooth germ contain only moderate amounts of the polysaccharide.

The developing stellate reticulum contains alkaline phosphatase, as in the molar tooth germ, but it is completely absent in the dental papilla.

As the tooth germ elongates to the bell stage, glycogen becomes more and more abundant in the cells of the external enamel epithelium, again predominantly on the labial side. It also accumulates in the peridental mesenchyme.

At the bell stage, the description of the histochemical properties of the different tissues as described by Pourtois (1961) and in part by other workers, is as follows:-

External enamel epithelium. Here, glycogen accumulates principally in the labial wall, where intensely positive staining for R.N.A. is found, and also alkaline phosphatase.

Stellate reticulum. The appearances of the stellate reticulum are as already described in the molar tooth germ.

Stratum intermedium. Likewise, the characteristics of this tissue are as in the molar tooth germ.

Internal/

Internal enamel epithelium. The staining reactions are similar to those of the molar. Engel (1948) states that the developing pre-ameloblasts in the middle one-third of the cell layer accumulate the most glycogen.

Dental papilla. Glycogen granules are invariably found in the incisor dental papilla, according to Engel (1948) and Glasstone (1958), whereas these same workers found it present only occasionally in the dental papilla of the molar tooth germ.

Mucopolysaccharides make their appearance intercellularly first of all at the anterior tip of the dental papilla, and spread toward the cervical loop region. Alkaline phosphatase is found in increasing quantities peripherally in the papilla as the tooth germ elongates.

iv) Stage of hard tissue formation.

The most detailed description of the histochemical changes found in the dental tissues during the formation of the hard tissues of the odontoblast and the ameloblast, is given by Pourtois in his paper of 1962.

At comparable stages of differentiation of the cells, the histochemical characteristics are identical in both the incisor and the molar tooth germ.

Stratum intermedium. These cells contain few glycogen granules before any hard tissue is formed, but when deposition of predentine begins, the glycogen granules are gradually lost (Ten Cate, 1962). As at the previous stage, their most significant characteristic is the high alkaline phosphatase content (Horowitz, 1942; Greep, Fisher & Morse, 1948; Dalq & Mulnard, 1953; Avery, 1954a).

Ameloblasts. These cells contain only little glycogen, and as in the stratum intermedium it is lost during amelo- and dentino-genesis (Horowitz, 1942; Bevelander & Johnson, 1946; Ten Cate/

Ten Cate, 1962). They do contain, and retain, high quantities of R.N.A. in the infra-nuclear zone (Wislocki & Sognnaes, 1950; Dalq, 1953; Johnson & Bevelander, 1954).

Predentine matrix. The predentine tissue at first stains positively for both neutral mucopolysaccharide (Bevelander & Johnson, 1955; James, 1957) and acid mucopolysaccharide (Wislocki & Sognnaes, 1950; Cederberg, 1951; Pincus, 1952; Hess & Lee, 1952 and Sasso & Castro, 1957). Pourtois (1962) states that it also contains alkaline phosphatase.

Odontoblasts. Bevelander & Johnson (1946) state that the odontoblasts stain heavily for alkaline phosphatase, but Pourtois (1962) found none present.

Dental papilla. The cells at the periphery of the pulp, lie in an intercellular matrix that stains positively for acid and neutral mucopolysaccharides. The cells themselves stain more positively for alkaline phosphatase than central cells in the papilla.

Gradually the thickness of the deposited dentine increases, and the features of the first formed dentine differs from that newly deposited. Maturing dentine at the stage of mineralisation contains neutral mucopolysaccharide and no acid mucopolysaccharide. Pinzon, Kozlov & Burch (1967) disagree, and state that mature dentine has a high acid mucopolysaccharide content - and again in opposition to the findings of Pourtois (1962) they think that the predentine contains less acid mucopolysaccharide than the maturing tissue.

Changes in the cells occur as the dentine matures. The odontoblasts lose their glycogen content as has already been described, and its concentration increases in the cells of the stratum intermedium. Ten Cate (1962), however, disagrees with Pourtois/

Pourtois, and has found the changes were of increased concentration in the pre-ameloblasts. Neither the odontoblasts nor the cells at the periphery of the pulp show any histochemical changes from the previous stage.

During elaboration of the enamel, which takes place shortly after the maturation of the dentine, few changes are seen in the histochemical picture. The stratum intermedium cells remain unchanged and the ameloblasts acquire positive staining properties for neutral mucopolysaccharide in the infranuclear zone (Engel, 1948). Bevelander & Johnson (1946) state that the glycogen content of the ameloblasts continues to decrease during amelogenesis. The dentine and predentine retain their previous characteristics, and now the odontoblasts, just like the ameloblasts, contain neutral mucopolysaccharides (Engel, 1948, agrees with Pourtois, 1962, on this point). The peripheral region of the pulp remains unchanged.

With maturation of the enamel, no change is seen in any of the cell layers described, except for the ameloblasts which now stain positively for alkaline phosphatase - as well as for neutral mucopolysaccharides and R.N.A. as before.

General description of the development of some derivatives of the mandibular arch.

In the young mandible, when the first arch of the aorta has been replaced by a network of capillaries throughout the mesenchyme, the former central position of the arch is occupied by a trail of closely packed mesenchymal cells gradually extending distally to fuse after several days, as the rostral process (Bhaskar, Weinmann & Schour, 1952). This trail of cells is the precursor of Meckel's cartilage (Hay, 1956; Milaire, 1959; Pourtois, 1961). Hyaline cartilage soon makes its appearance.

The dentary bone is formed around Meckel's cartilage, principally on the lateral and superior aspects. It is formed mainly by membranous ossification (Hay, 1953, 1961; Milaire, 1959), but part stems from Meckel's cartilage which is obliterated by waves of endochondral ossification which spread proximally and distally from the region of the mental foramen (Bhaskar, Weinmann & Schour, 1952; Hay, 1956, 1961; Friant, 1958; Dreyer, 1964). Secondary cartilages develop near the posterior edge of the lateral plate of the dentary, precursors of the condylar and angular cartilages, to complete the general outline of the mandible (Hay, 1961).

The mandibular branch of the Vth. (Trigeminal) cranial nerve enters the mandibular arches at the same time, and very close to, Meckel's pro-cartilage. The dentary develops around it.

Development of the salivary glands of the mouse has been described in detail by Borghese (1950a). The submandibular and major sublingual glands develop from separate epithelial invaginations into the mesenchyme on either side of the developing tongue. The submandibular gland opens into the medial of the two ducts which develop from the invaginations, and develop slightly in advance/

advance of the sublingual glands. The epithelial invaginations elongate as solid cords, branching profusely into a dense mesenchymal capsule. Acini develop peripherally on the branches. At first, the epithelial cords and acini are solid, but the main ducts soon become hollow, closely followed by the smaller, narrower branching ducts, and finally by the acini. Both glands develop within the same capsule, the submandibular gland enlarging and migrating to overlie the hyoid bone, extending to the sternum and clavicles, and the smaller sublingual gland lies lateral to it. Neither are mixed glands: the submandibular gland is a serous gland, the sublingual, a mucous gland (Green, 1966).

The thyroid gland arises from tissue between the first and second branchial arches. Its normal development is relevant to this study because thyroid acini are encountered from time to time in cultured tissues from mandibles of very young embryos where the tissue was too small for successful separation of mandibular derivatives. De Haan & Ursprung (1965) could find no references specifically to normal thyroid gland development in the mouse at its early stages. Except for two articles in Japanese written before this date, the deficiency has not been remedied. Normal development of the thyroid will therefore be covered in the results of this section.

METHODS AND MATERIALS.

The experimental animal chosen was an inbred-derived strain (Green, 1966) of a brown agouti mouse bred for many years at the Department of Zoology, University of Edinburgh. For convenience of comparison with other strains, it will be referred to throughout the work as the EZL (Edinburgh Zoological Laboratory) strain. An inbred strain was avoided, because of the difficulty of obtaining a sufficient number of litter mates for control and experimental purposes from the small litter sizes expected in such cases (Green, 1966). The average litter size of the EZL strain is 10, and the gestation period is 19 days.

1. Methods of Breeding Employed.

Method 1.

Monogamous pairs were placed in each cage. The females were examined each morning during the experimental period for the presence of vaginal plugs. 86% of mice with plugs became pregnant. The day of appearance of the plug was taken as day 0 in the gestation period of the embryo.

Method 11.

The Whitten Effect was utilised (Whitten, 1956a, 1956b, 1959), the method being as modified by Ross (1961, 1962). Females were exposed to the olfactory stimulus of a corralled male for 3 days, and were then mated with this same or other males. Instead of the random number of matings expected over the next four nights (Parkes, 1928) the olfactory stimulus of the male had synchronised oestrus in the females, increasing the number of matings during the first night after release.

2./

2. Methods of Examination of Material.

a) Macroscopic examination.

The external features of the embryo at each day of gestation age were noted, with the intention of using the information in future experimental work as a rapid check on the exact age of any embryo in which it might be in doubt. The features noted were approximate crown-rump (C.R.) length, the number of somites visible, the stage of development of the eye, ear, whiskers, vascular system (the heart in particular) anterior and posterior limb buds, tail and state of closure of the gut tube. A close correlation was found between these observations and those of Gruneberg(1943). The degree of fusion of the right and left mandibular arches was noted in the younger embryos.

b) Microscopic examination.

Histological and histochemical methods for light microscopy.

i) Fixation. The embryological tissues were routinely fixed in Zenker's Fluid, containing 0-3% acetic acid depending on the age of the embryo, for 3 minutes - ½ hour for isolated mandibles and for 3 - 12 hours for whole embryos. They were then transferred to Zenker's Fluid alone for a further 1 - 2 hours (Hay, 1961; McLoughlin,1959). The fixed tissues were washed in several changes of tap water for 2 - 3 hours and dehydrated. Other fixatives used occasionally on post-natal material were neutral formalin (Lillie,1954) and Heidenhain's Susa Fluid(Carleton & Drury, 1957).

ii) Dehydration and embedding. Fixed and washed tissues were dehydrated in a graded series of alcohols, 30-98 %, except for those tissues fixed in Heidenhain's Susa Fluid, which were transferred directly to 98% iodine alcohol. The mercuric deposit from Zenker's Fixative was removed by the addition of iodine to the 70% or 80% alcohols. The tissues were then transferred to either absolute alcohol, or more commonly to several changes of carbol xylol.

The/

The clearing agent of choice was methyl benzoate for 6-24 hours, though cederwood oil and benzene were used on occasion. The tissues were embedded in paraffin wax or fibrowax, M.Pt.56-58°C. and embedded in watch glasses or in Leuckhart's L-shaped moulds.

iii) Sectioning. Serial sections were cut at 5 μ in both coronal and sagittal planes at each day of age. Few sections were placed on each slide, thus increasing the chance of any tooth germ being stained by as many different methods as possible.

iv) Staining. The stains used routinely were:-

1) Harris's Haematoxylin and Eosin (Carleton & Drury, 1957) - the most useful general purpose stain, which allowed the rapid identification of epithelial and mesenchymal tissues.

2) Heidenhain's Azan Method (modified from Lillie, 1954). Young collagen fibrils were identified by their intense blue staining.

3) Periodic acid - Schiff technique (P.A.S.) counter-stained with Mayer's Haemalum (Lillie, 1954; Pearse, 1960; Haemalum, Carleton & Drury, 1957), or P.A.S. technique counter-stained with Heidenhain's iron haematoxylin, Orange G/Picric Acid, (McLung, 1950). Purplish-red shades of staining indicate precise histochemical reactions between the stain and poly-saccharides, neutral mucopolysaccharides, nuco- and gluco-proteins, glycolipids, unsaturated lipids and phospholipids. Lipids, in all cases in this study, were inevitably removed during preparation.
To differentiate between polysaccharide and neutral mucopolysaccharide distribution, slides adjacent to these stained with P.A.S. were subjected to treatment with/

with diastase (1% solution at 37°C.). The diastase removed the polysaccharide glycogen, but did not affect the neutral mucopolysaccharides. Celloidin was used to prevent enzyme digestion separating the sections from the slide.

To test the efficiency of the P.A.S. stains, control sections of known tissues were always stained at the same time as experimental sections.

Test sections used were chick embryo leg and mouse intestine. In chick embryo leg glycogen is present in the periderm layer of the epithelium, glycoprotein in the basement membrane and neutral mucopolysaccharide in the cartilage matrix. In the mouse intestine the epithelial mucin served as a strongly P.A.S.-positive test material. The staining reactions of the experimental slides were accepted only if the test slides stained as expected.

4) Lillie's Allochrome Connective Tissue Method (Lillie, 1954). Lillie's method combines the P.A.S. technique with Azan staining for young collagen fibres and Weigert's iron haematoxylin for good nuclear detail. Its use enabled as much histological and histochemical information as possible to be derived from small volumes of tissue. Diastase treatment was carried out on adjacent sections, as with the P.A.S. technique to distinguish neutral mucopolysaccharides from polysaccharides. Striated muscle fibres and thyroid colloid were also well demonstrated by this procedure.

5) Alcian Blue Method for acid mucopolysaccharides. (Modified from Lison, 1954 and Pearse, 1960). The counterstain used was Mayer's Haemalum. As with the P.A.S. technique, the efficacy of the Alcian Blue stain was/

was checked by the use of test sections of chick embryo leg and mouse intestine. The intestinal mucin, and the cartilage matrix stained an intense clear turquoise blue, due to their known acid mucopolysaccharide content. Test sections were included with each series of experimental material during the staining procedure.

Stains used occasionally were:-

1) Toluidine Blue, for acid mucopolysaccharides (Pearse, 1960). Metachromasia is exhibited by acid mucopolysaccharides stained with toluidine blue. Test slides of chick embryo leg and mouse intestine were used as before.

2) Methylene Blue for acid mucopolysaccharides (Pearse, 1960). The acid mucopolysaccharides were identified by their ability to bind methylene blue below pH4. The sections were examined under water.

3) Verhoeff's Elastic Tissue Stain (McLung, 1950). Elastic tissue fibres in the mesenchymal tissues were identified by their black staining.

4) Masson's Trichrome Stain for connective tissues (Carleton & Drury, 1957). Collagen fibrils were identified by their blue colouration.

5) Vicker's Modification of the Elftmann method for the Golgi apparatus (Cambridge University Physiology Part II Practical Histology Notes, 1963).

Schedule:-

1) Fix the tissues in 2% silver nitrate in 10% formalin. Adjust the pH to approximately 4-5 with a few drops of acetic acid followed by 5% sodium acetate.

2)/

2) Rinse in distilled water for 5 minutes.

3) Reduce in 1% hydroquinone in 10% formalin for 3 hours.

4) Complete fixation for a total of 24 hours with 10% formalin.

5) Wash, dehydrate via alcohols, clear and embed in the usual way. Cut sections and mount.

6) Bring sections to water.

7) 'Tone' in gold chloride for 3-4 minutes, 'fix' in 5% sodium thiosulphate and wash thoroughly. Counterstain with P.A.S. and Toluidine Blue or Alcian Blue and Neutral Red. Dehydrate, clear , mount in balsam.

Structures other than Golgi apparatus stain with this method, and care is essential in interpretation. The Golgi apparatus stains black.

RESULTS.

TABLE 1.

DEVELOPMENT OF MANDIBULAR ARCHES FROM THEIR FIRST APPEARANCE TO THE DEPOSITION OF ENAMEL MATRIX IN INCISOR, FIRST, AND SECOND MOLAR TOOTH GERMS.

TABLE I.

DEVELOPMENT OF THE MANDIBLE IN VIVO

Age of Embryo	Number of Embryos Examined	Approximate C - R Length	Figure Number	Morphological Features of Mandible			Histological Features of Mandible			HISTOCHEMICAL FEATURES OF THE MANDIBLE			
										Glycogen	Neutral Muco-polysaccharide	Acid Muco-polysaccharide	Collagen Fibril Formation
days				INCISOR TOOTH GERM	MOLAR TOOTH GERM	OTHER FEATURES	INCISOR TOOTH GERM	MOLAR TOOTH GERM	OTHER FEATURES				
8	13	1.5 mm.	Fig. 5	Mandibular arches unfused. No blood or nerve supply.			'Oral' epithelium 2-3 cells thick - basal cells columnar, upper layers flattened. Mesenchymal cells undifferentiated.			None	+ve basement membrane (bm)	+ve at bm	None
9	4	2.5 mm.	Figs. 6-8	Arches unfused. Blood supply is 1st. arch of aorta.			Primordium of Meckel's cartilage present as proximal condensations of mesenchymal cells, central in arches. Thyroid gland appears as an epithelial invagination (10-15 cells) in mid-line between 1st. and 2nd. branchial arches, above the developing heart. There is no mesenchymal condensation (Fig. 43). A region of cell death is present inferiorly in the arches.			+ve in cells of Meckel's cartilage anlage. +ve in cells of the epithelium and mesenchyme of the thyroid gland anlage.	As before	+ve intercellularly in mesenchyme.	None
10	9	4 mm.	Figs. 9-11	Arches fusing. Mass of capillaries has replaced 1st. arch of aorta.			Mesenchymal cells in oral half of mandible are condensed and have densely basophilic cytoplasm. Anlage of Meckel's cartilage now extends distally about a quarter of the length of the arch.			As in 8-9 day embryo.	As in 8-9 day embryo.	As in 8-9 day embryo.	As in 8-9 day embryo.
11	13	6 mm.	Fig. 12	Arches fused. Inferior alveolar nerve now present proximally.			First appearance of dental lamina present as three separate ingrowths of epithelium, one proximal in each arch, one distal in the mid-line. Chondroblasts present proximally in Meckel's cartilage. Epithelial invagination for thyroid gland has lost connection with oral epithelium and lies just above the 3rd. arch of the aorta.			+ve in epithelial cells at junction of maxilla and mandible.	+ve intercellularly in oral half of mandible.	As before.	First appearance next to bm.
12	7	8 mm.	Fig. 13				First appearance of epithelial invaginations and mesenchymal cell condensation for submandibular and sublingual salivary glands (Fig. 38).			+ve in surface layers only of dental lamina. +ve-chondroblasts. +ve-intercellular mesenchyme centrally in arches.	As in 11-day embryo.	As in 11-day embryo.	As in 11-day embryo.
13	7	8.5 mm.	Figs.15-17	-	-	-	Mesenchymal condensations localising beneath each dental lamina invagination.		Each salivary gland represented by epithelial stalk and terminal bud within common mesenchymal cell condensation. Thyroid gland epithelium now extends round pharynx - cells have densely staining nuclei, little cytoplasm and are arranged in clumps. Matrix of dentary appears.	+ve-(intensely) in lip furrow band. +ve in oral epithelial +ve halo intercellularly round proximal dental laminae.	+ve intercellularly throughout mesenchyme	Unchanged	+ve intercellularly at site of dentary formation lateral and superior to Meckel's cartilage.
14	8	10.5 mm.	Figs.18-21	Enamel organ (E.O.) elongating proximally	M_1 is at cap stage	Meckel's cartilage fusing in mid-line. Dentary is forming and extending towards M_1. Submandibular duct structure developing. Fig. 40.	Dense blood capillary supply around tooth germ.	Dental follicle now present as mesenchymal cell condensation around E.O.	Thyroid epithelial cells now in cords invaded by vascular sinuses.	+ve intercellularly and intracellularly as halo at distance around E.O.	No significant change.	No significant change.	1st. appearance of collagen in D.P. of incisor and molar tooth germs.

Age of Embryo	Number of Embryos Examined	Approximate C - R Length	Figure Number	Morphological Features of Mandible			Histological Features of Mandible			Histochemical Features of the Mandible			
				Incisor Tooth Germ	Molar Tooth Germ	Other Features	Incisor Tooth Germ	Molar Tooth Germ	Other Features	Glycogen	Neutral Muco-polysaccharide	Acid Muco-polysaccharide	Collagen Fibril Formation
days													
15	6	12.5 mm.	Figs.22-24			Rostral process forming.	1st. appearance of pre-ameloblasts.	1st. appearance of pre-ameloblasts. 1st. appearance of intercellular material in S.R. Dental lamina present for M_2.	Secondary cartilages present in dentary for condylar and coronoid processes.	+ve-cells of dental lamina. +ve-cells of I.E.E.	More dense intercellularly in D.P. than in surrounding mesenchyme.	More dense intercellularly in D.P. than in surrounding mesenchyme.	No significant change.
16	5	14.5 mm.	Figs.25-27	-	Cusps L_1B_1: L_2B_2 present.	-	1st. appearance of odontoblasts.	1st. appearance of odontoblasts.	1st. appearance of stratum granulosum in oral epithelium. 1st. appearance of endochondral ossification in Meckel's cartilage.	+ve-in S.R. Fig.46. +ve-cells of I.E.E.	+ve in S.R. of M.	+ve in S.R. -ve in dental lamina.	Gradual increase in density and calibre of fibres - especially at incisal tip of D.P.
17	8	18 mm.	Figs.28-30	-	Cusps of M_1- L_1B_1:L_2B_2: L_3B_3. M_2's development 3 days behind that of M_1.	Salivary gland acini develop Fig. 41. Vesicle formation in thyroid gland. Fig.44.	1st. appearance of predentine.	Well differentiated S.R. and S.I.	1st. appearance of keratin in oral epithelium. 1st. appearance of calcification in dentary. 1st. appearance of colloid in thyroid gland.	+ve-in S.R. +ve-in D.P. of M_1 and incisor -ve-dorsum of tongue.	+ve in S.R. of M.	+ve in S.R. of M_1 and incisor.	Fibres more dense in D.P. of incisor than in surrounding mesenchyme.
18	4	19 mm.	Figs.31-33	-	-	-	Dentine matrix extends down $\frac{2}{3}$ of developing tooth germ.	Red blood cells present in S.R. of M_1.	1st. appearance of resorption vesicles in thyroid gland.	+ve-in E.E.E.(fine granules) +ve-in S.R. +ve-in S.I. +ve-in I.E.E.(sparse) +ve-in D.P.-intense in some places	+ve in S.R. - more intensely staining.	+ve in S.R. - more intensely staining.	-
19 Birth	4	21 mm.	Figs.34-36	-	Completed crown pattern of M_1. L_1B_1 : L_2B_2 : L_3B_3:4	-	1st. appearance of enamel matrix. Maturation of dentine matrix begins.	1st. appearance of predentine on cusps L_1L_2 of M_1.	-	+ve-pre-ameloblasts at tips of cusps. +ve-S.I. Disappearing in S.R. Increasing in E.E.E.	More intensely staining in S.R.	More intensely staining in S.R.	Fibrils increase in calibre.
1 day after birth	5	-	Figs.37-38	-	M_2-Cusps : L_1B_1 : L_2B_2: L_3B_3.	-	-	1st. appearance of predentine in M_2.	-	Dense granules in D.P. of M_1. Fine granules in D.P. of incisor.	-	-	-
2 days after birth	4	-	-	Now extends proximally to level of cusps of M_2.	-	-	Calcification of dentine well advanced.	1st. appearance of enamel matrix in M_1.	Resorption vesicles obvious peripherally in thyroid colloid.	-	-	-	-
3 days after birth	2	-	Fig. 39	-	-	-	-	1st. appearance of enamel matrix in M_2.	-	-	-	-	-

DISCUSSION

By studying the different stages of mandibular development *in vivo*, and relating them to the gestation age of the embryo, a useful set of standards is provided which has two main applications.

Firstly, it can be used to gauge the development of tooth germs grown *in vitro*, as is done in Part II of the work.

Secondly, it can be used to compare early tooth development in the EZL strain of mouse with that of other strains as reported in the literature.

The work presented here not only records the development of the teeth in a strain not previously studied, but it allows a critical examination of the published work on other strains. In particular it has allowed critical comment on the interpretations placed by different workers on the spatial distribution of cells and intercellular material during the intense activity of early tooth development. As mammalian embryos are limited in their ability to withstand experimental intervention and continue to develop, our knowledge of the inductive influences of the epithelium or the mesenchyme during the organogenesis of the tooth in its early stages, has been based mainly on assumptions made after examining the distribution of cells, intercellular and intracellular materials from normal histological and histochemical preparations of the tissues.

Morphological Features of Development.

The morphological development of the main cusps of the teeth of the EZL strain is similar to that of other strains described in the literature, except for that of Gaunt's inbred strain where cusp B_1 in M_2 is missing. In the EZL strain, M_1 is composed of cusps L_1 B_1 : L_2 B_2 : L_3 B_3: 4 and M_2 is composed of B_1 : L_2 B_2 : L_3 B_3 : 4.

Histological/

Histological Features of Development.

As far as the actual sequence is concerned, histological development shows few major differences from that of other strains. The time scale, however, differs. Inthe EZL strain, the first appearance of the dental lamina is seenon the 11th day in all embryos examined. This is the same age at which the lamina appeared in the strain of mouse used by Pourtois (1961), but differs from that found in other strains (Gaunt, 1955; Fisher, 1957; Hay, 1956; Cohn, 1957 and others). Later features of development inPourtois' strain are not similar to those of the EZL strain. For example, there is no diastema in the dental lamina between the incisor and molar regions in the strain used by Pourtois whilst there exists a clearly defined one in the EZL strain. Timing of the appearances of the cusps of M_1 is similar in the two strains at the 16-day stage when cusps L_1 B_1 : L_2 B_2 are present, but thereafter cusp development is delayed in Pourtois' strain.

The results agree well with Grüneberg's conclusions (1943) that within each strain of mouse there is very good correlation of timing of development between embryos, and with Fisher (1957) who, after a detailed comparison of the Bristol strain and Gaunt's strain of mouse, noted that 'differences of rate, but not in sequence of development between different strains of the same species is common'.

By analogy with amphibian teeth where there appears experimental proof that tooth formation is the result of a secondary induction involving different germ layers, it has been assumed that mammalian tooth formation is based on a similar phenomenon. Consequently, significance has been placed on the distribution of certain histological and histochemical features which seem to be placed advantageously to influence surrounding tissues. These features are:- a) the blood supply to the mandible/

mandible; b) the presence of the basement membrane between the epithelium and the mesenchyme in odontogenic regions; c) the condensation of mesenchymal cells beneath the developing dental lamina, and d) the presence in the mandible of cells identified histochemically as of neural crest origin. The validity of these assumptions is considered.

a) The blood supply.

Milaire (1959) was of the opinion that the future morphology of the tooth is determined by initial variations in regional metabolites in odontogenic areas - notably alkaline phosphatase, ribonucleic acid and glycogen. The variations were thought to be dependent on the establishment of the vascular system.

As we have seen, the first arch of the aorta which supplies the mandibular arch is replaced within a few hours by a trellis-work of capillaries. Gaunt (1959) states that there is a capillary vascular system underlying the future position of the tooth before there is any evidence of ectodermal thickening. On the other hand, Milaire (1959) noted that the capillaries were apparently distributed at random throughout the mesenchyme of the mandible.

Pourtois (1961) passed no comment on the capillary distribution at the early stages, but did note that at a slightly later stage, when individual epithelial thickenings on the dental laminae for molar and incisor teeth are present (12 days) the capillaries surrounding the dental zones are well known to be dilated. They are then placed on each side of the molar lamina and on the labial side of the incisor lamina. The blood vessels remain on the labial side of the incisor tooth germ, whereas the R.N.A.-rich cells, also surrounding the lamina at the early stages, move lingually. This separation allowed Pourtois (1961) to differentiate between the activities of the two significant factors. He/

He decided that the blood supply played no part in prompting epithelial thickening, but that it was responsible for the greater rate of growth of the labial surface of the tooth germ.

This observation is of interest in the light of similar deductions made on the development of hair follicles - structures very similar in early developmental pattern to teeth. Hardy (1952) found no dense blood supply around developing follicles in mice before birth, thus signifying lack of participation in the induction of the hair follicle - but she did find a dense supply to the shaft of the developing hair at the time of maximum growth, which led her to think that the blood supply was responsible for the support of intense mitotic activity at this time.

Study of the vascular supply to developing hair has also prompted the idea that where there is a limited supply of energy distributed by the vascular system, tissues enter into competition for its utilization. In mice, where periodic waves of new hair growth begin mid-ventrally and pass dorsally (Bullough, 1952), a rich capillary network opens in the dermis beneath the advancing wave (Haddow, Elson, Roe, Rudall & Timmis, 1945). Several observations seem to support the concept of tissue competition for the available energy : there is a reductionin growth (i.e. mitotic) rate in the hair follicles overlying mammary glands active during lactation (Strangeways, 1935; Durward & Rudall, 1945) : epidermal warts in the wave of actively growing hair follicles shrink and blanch (Mottram, 1945) : the finest wool develops in the densest fleece (Fraser, 1951).

No strictly comparable deductions have been made from correlating blood supply and tooth development, but the observations of Grüneberg (1965) are not at variance with the idea of tissue competition controlled by energy requirements. Whilst studying intra-specific genetic variabilities in the mouse, he noted that the size of M_1 appeared to be directly under genetic control. Studying/

Studying different mutants, he found that where M_1 is markedly reduced in size 'weakened competition releases growth potentialities in M_2 and M_3 which in extreme cases may grow beyond the size found in the normal mouse'. Further studies of blood supply and tooth size or number would be of great interest.

My own observations confirm that the first arch of the aorta is quickly replaced by a capillary network in the mesenchyme. An increased blood supply to the odontogenic areas is apparent early in development. It is definitely localised at the time of the individual epithelial thickenings on the laminae, but as development is so rapid at the early stages, it is almost impossible to tell whether the localisations of blood capillaries are present before the thickenings appear, or are merely concomitant with them. Straight-forward examination of histological material would not seem to be a sound basis for deciding whether the blood supply plays a part in inducing odontogenesis, or simply supports future development.

b) The basement membrane.

In his study of the development of the mandible with the light microscope, Pourtois (1961) noticed that for a period of twenty-four hours immediately prior to the appearance of the dental lamina, the basement membrane between epithelium and mesenchyme in the presumptive dental zone disappeared. Milaire (1959), also using light microscopy, noted a similar finding at the same stage of development, but did not specify which sites in the branchial arches were involved.

Bearing these facts in mind, I made a close study of the basement membrane in odontogenic zones, staining the membrane with periodic-acid Schiff reagents to reveal glycoproteins (Gersh & Catchpole, 1949). I found that at no time did the basement membrane seem to disappear.

Milaire/

Milaire (1959) and Pourtois (1961) believe that when the basement membrane disappears, macromolecules are able to pass freely between the epithelium and mesenchyme. The loss of the basement membrane, by providing such a ready means of induction, is thus a potentially important stage in future development. Electron microscopic studies of odontogenic areas are necessary to prove, beyond dispute, the presence or absence of the basement membrane.

Of relevance, is the study of Jurand (1965) of the ultrastructure of the apical ectodermal ridge of the developing mouse limb. He found the basement membrane to be present at all stages of development. Its presence, therefore, in this region at least, does not impede the complex patterns of induction known to take place.

c) The mesenchymal condensation.

The influence exerted on early odontogenesis by the local vascular system, by neural crest cells, or by the presence of the basement membrane is assumed to be active before the onset of morphological changes. When morphological changes do appear, the epithelial invagination is invariably accompanied by the mesenchymal condensation. Accordingly, the belief has arisen that even at this relatively late stage, development must be synchronised by some means of communication between the tissues.

In deciding whether the mesenchymal condensation is present in response to epithelial activity, or is intrinsic to activity on the part of the mesenchyme itself, two relevant considerations arise:- a) the order, in time, in which the mesenchymal condensation and the epithelial invagination emerge - the assumption being that whichever appears first would be the active partner, and b) whether the mesenchymal condensation stems from mitosis *in situ*, or by an influx of cells from its periphery.

Thomas/

Thomas stated as early as 1925 that mesenchymal activity in tooth development is not seen before epithelial activity. More recently (1953), however, Lefkowitz, Bodecker & Mardfin noted that mesenchymal cells proliferated simultaneously around the developing lamina. Milaire (1959), despite a very detailed description of tooth development at this stage, makes no mention of it, and Pourtois (1961) merely states that at the stage where individual tooth germs form on the dental lamina, the cells of the underlying mesenchyme are compressed by the epithelial growth.

My results show that: a) the cells beneath the early dental lamina are no more condensed than in other sites adjacent to the oral epithelium; b) that once individual tooth germs are forming on the lamina, then there is an indication of closer packing of surrounding cells, and c) that by the time the tooth germ has reached the cap stage of development, there is a pronounced localised condensation of mesenchymal cells. My results, therefore, shed no light on which tissue is taking an active part in influencing tooth development.

As for mitotic activity within the condensation - no accurate reports have been made. Tonge (1951; 1953; 1966); Lefkowitz, Bodecker & Mardfin (1953); Pourtois (1961) noted that mitotic figures appeared to be as numerous in the mesenchyme as in the invaginating epithelium. Cohn (1957) suggested that initially numerous mitotic figures are to be seen making up the condensation, but then the cells aggregate around the developing lamina.

That the mesenchymal condensation in tooth development may not be as simple as cursory examination of histological material suggests, is hinted at by the studies of Wessells (1965) on the mesenchymal condensation of the feather germ - a structure similar in early development to teeth. Using tritiated thymidine and uridine, he found that mitotic figures are arranged around the periphery of the aggregation, and that R.N.A. is synthesised by the mesenchymal cells both during the proliferative and the non-proliferative/

proliferative stages. Similar studies on tooth development might throw some light on the activities of the mesenchymal cells in odontogenesis.

d) The neural crest cells.

Several reports (for example,Sellman, 1946; de Beer, 1947; Hörstadius, 1950; Detwiler & Levy, 1952; Levy, 1954; Avery, 1954b; Chibon, 1967) stress that for amphibian teeth to develop, three tissues are essential - oral ectoderm, oral endoderm and cells of the neural crest (ectomesenchyme). Amphibian eggs are eminently suitable for experimental manipulation, and the role of the neural crest cells has been verified by numerous experiments involving vital staining, isotopic labelling, extirpation, transplantation of neural crest cells, and by the formation of chimaeras where cellular details are different and detectable.

It is unfortunate that the mammalian embryo can not be subjected to the same procedures. Although the role of the neural crest cells in mammalian tooth development has been the subject of controversy for a number of years, no report of any experimental work is to be found.

However, Pourtois (1961) states that his studies prove without doubt, that ectomesenchymal cells are responsible for inducing odontogenic activity in the oral ectoderm of the mouse. His proof is based on the identification of certain cytochemical features which he believes differentiates neural crest cells from the surrounding mesenchymal cells. Both he, and Milaire (1959) identified the ectomesenchyme as a mass of cells, rich in ribonucleic acid and alkaline phosphatase. The mass can be traced from the region of the Gasserian ganglion, passing forwards to be diverted partly into the maxillary process, and partly into the mandibular arch. In the mandibular arch one part of it differentiates into Meckel's cartilage, the other part lies in close proximity to the dental lamina region, and the dental papilla and odontoblasts are thought to differentiate from it.

Convincing/

Convincing as these ideas appear, there are several drawbacks to their unqualified acceptance. Milaire (1959) notes that at an early stage (8 days), there is confusion and difficulty in tracing the cells from the Gasserian ganglion into the mandibular arch. The presence of ribonucleic acid in cells cannot be attributed wholly to their ectomesenchymal origin. It has long been known (Brachet, 1950; Davidson, 1965) that cells actively synthesising protein - as we could expect in the rapidly growing and differentiating mandible - contain large quantities of ribonucleic acid. In fact, Gaunt and Miles (1967) point out that gradients of ribonucleic acid are regarded by many workers as an indication of gradients of morphogenetic or metabolic activity. For convincing proof using cytochemical means, a more specific metabolic substitute for ectomesenchyme (e.g. phenylalanine - Saxén & Toivonen, 1962), would have to be tested for.

Histochemical Features of Development.

A comparison of the histochemical features of tooth development in the EZL and other strains, is not as straightforward as the comparisons already made of morphological and histological development.

In all strains examined, including the EZL strain, glycogen is present in the oral epithelium (Bradfield, 1951; Chiquoine, 1957; Milaire, 1959; Pourtois, 1961; Forter & Lefkowitz, 1965). It is found in the differentiated pre-ameloblasts (Bevelander & Johnson, 1946; Engel, 1948; Glasstone, 1958; Pourtois, 1961) and during hard tissue formation, it is lost from the ameloblasts (Horowitz, 1942; Bevelander & Johnson, 1955; Pourtois, 1961).

I agree with the majority of workers that at the bell stage of development, the stellate reticulum contains glycogen (see Ten Cate, 1957; Glasstone, 1958 and many others cited in the introduction). Horowitz (1942) seems to be the only author to disagree. The majority of workers (e.g. Glock, 1940; Horowitz, 1942; Bevelander & Johnson, 1946; Pourtois, 1961) also agree with my finding that the/

the cells of the internal enamel epithelium before differentiation into pre-ameloblasts, contain glycogen. Sundberg (1924) disagrees.

Reports in the literature of the presence of glycogen in the dental papilla give rise to a more confused picture. Glycogen was found there invariably by Wislocki, Singer & Waldo (1948), Wislocki & Sognnaes (1950) and Russell (1967). Engel (1948) and Glasstone (1958) found it in the dental papilla only occasionally, although on the other hand, Glock (1940), Horowitz (1942) and Bevelander & Johnson(1946) found it absent. Pourtois (1961), examining mouse dental papilla, and Russell (1967) with bovine dental papilla, found that the amount of glycogen present increased during development in the central zones. Engel (1948) and Glasstone (1958) found glycogen invariably present in the dental papilla of the incisor tooth germ, although the two workers found it positive only occasionally in the papilla of the molar tooth germ.

My results showed that in the EZL strain, glycogen was invariably present in the dental papilla of both molar and incisor tooth germs. It appeared first when the tooth germ was in the early bell stage, and was uniformly distributed.

There is little disagreement about the distribution of neutral mucopolysaccharide (N.M.P.S.) in the developing tooth - and my results support almost all other reports.

I found N.M.P.S. present throughout the oral half of the mandible from the 11-day stage onward, i.e. from the time of the first appearance of the dental lamina. At the bell stage of development, the papilla stained more intensely for the N.M.P.S. than elsewhere in the undifferentiated mesenchyme of the mandible. I could find no definite signs of localisation within the papilla, but Pourtois (1961) found it in highest concentration in the central zones, and Wislocki, Singer & Waldo (1948), Wislocki & Sognnaes (1950), Cederberg (1951) and Bevelander & Johnson (1955), found the highest concentration at the periphery. In the elongating incisor/

incisor tooth germ, I agree with Pourtois (1961) that N.M.P.S. concentrates first at the incisal tip, then extends gradually towards the cervical loop.

My results of the N.M.P:S. distribution in the later stages of tooth development agree with all other reports: N.M.P.S. is found in both predentine and maturing dentine (James, 1951; Bevelander & Johnson, 1955; Pourtois, 1962), in the infra-nuclear zone of the ameloblasts during enamel matrix production and later in the odontoblasts (Engel, 1948; Pourtois, 1962),

Acid mucopolysaccharide (A.M.P.S.) is known to be found in many identical sites to N.M.P.S. (Curran, 1961), and this is true of its distribution in the developing tooth germ. Its concentration in the dental papilla is found by Pourtois (1961) and myself, to be higher than elsewhere in the connective tissue of the mandible.

A.M.P.S. is a striking histochemical feature of the enamel organ at the bell stage of development (Bevelander & Johnson, 1955; Butler, 1956; Pourtois, 1961; Baratieri, 1965 and myself). My observation that it is also present in predentine is in agreement with all other reports (Wislocki & Sognnaes, 1950; Cederberg, 1951; Pincus, 1952; Hess & Lee, 1952; Sasso & Castro, 1957, and Pourtois, 1962).

As with the histological features of the developing mandible, so with histochemical features - assumptions have been made as to the functions of the features examined:-

a) Glycogen.

Those who have investigated its distribution inodontogenic tissues (Dalq, 1953; Milaire, 1959; Pourtois, 1961; Sasaki, 1967) all agree that glycogen, a polymer of glucose, is present as a source of phosphate-bond energy for future proliferation and differentiation. The observation of Moog & Wenger (1952), Ten Cate (1962), Matthiesen(1963) and Sasaki (1967) that there is a gradual decrease in glycogen content with progressive development of/

of the tooth germ, supports the theory.

Glycogen first appears in the oral epithelium in odontogenic areas after the disappearance in identical sites of alkaline phosphatase, leading Pourtois (1961) to believe that the phosphatase is concerned in glycogenesis there. Moog (1944) states less specifically that, in general, phosphatase is concerned with cell division.

In later development of the tooth germ - the disappearance of glycogen from the stellate reticulum, stratum intermedium and internal enamel epithelium, was traced by Kroon (1952) and Ten Cate (1962) to be co-incident with the onset of enamel matrix production, and they agree with Moog & Wenger (1952) that the role of glycogen in amelogenesis is as a source of hexosephosphate esters. Sasaki (1967) states in a less specific manner that it plays a part in hard tissue formation.

In the dental papilla, and the stellate reticulum, where neutral and acid mucopolysaccharides form, glycogen also disappears and Pourtois (1961) believes that those compounds have developed at the expense of the glycogen. Pourtois (1961) interpreted the presence of alkaline phosphatase in these sites and at this stage of development, as a factor favouring the synthesis of mucopolysaccharides from glycogen.

b) Neutral mucopolysaccharide.

Matthiesen (1963) and Pourtois (1961) as stated, have associated the disappearance of the glycogen from the dental papilla and enamel organ with the appearance of the neutral mucopolysaccharide (N.M.P.S.). Moog & Wenger (1952) presented evidence that neutral mucopolysaccharide was associated with cytoplasmic sites of high alkaline phosphatase activity in all tissues examined - even in embryonic tissues where their concentration is relatively poor.

As/

As to the reason why the concentration of N.M.P.S. in the dental papilla appears to be higher than elsewhere in the connective tissue of the mandible, no-one as yet has hazarded an explanation. Laurent (1968) exphasises that in general, the presence of polysaccharides in the ground substance of connective tissues will affect the partition of various proteins between these tissues and the blood. He thought, as does Moog & Wenger (1952), that the polysaccharide acts as a sieve for the transport of macromolecules - or as a cyto-chemical structure - the macromolecules passing either by diffusion through the polysaccharide components of the mesenchyme, or by solvent flow between the compartments. He also pointed out that the polysaccharides had the ability to decrease the solubility of the macromolecules and could cause precipitation in the connective tissue, e.g. collagen fibre formation, or mineralization of bone.

In such an actively forming organ as the tooth germ where complex matrix production must be carried out, and where in particular collagen fibril formation is essential to the matrix formation, it can be readily understood that N.M.P.S. could have a very important role to play in its metabolism.

c) Acid mucopolysaccharide.

Acid mucopolysaccharides (A.M.P.S.) are a striking feature of the epithelium-derived stellate reticulum, and also in the mesenchyme-derived dental papilla. Symons (1967) states that due to the conflicting findings regarding the action of hyaluronidase, it is at present impossible to identify precisely the sulphated acid mucopolysaccharides in the pulp. Little more evidence is available for the epithelial acid mucopolysaccharide, except that its intense staining with Alcian blue indicates a liklihood of the presence of more carboxyl than sulphate groups (Quintarelli, 1968).

The hygroscopic nature of the A.M.P.S. has led to speculations concerning its function in the stellate reticulum. Gulat (1936) thought that the stellate reticulum tended to maintain the shape of the/

the dental cap during development, Avery (1951) that it ensured the requisite space for crown development within the tooth follicle. Santoné (1936) assumed that it protected the internal enamel epithelium by acting as a mechanical buffer, and Butler (1956) reasoned that the osmotic pressure could cancel out the counter pressure of the dental papilla and thus allow the internal enamel epithelium to fold according to its intrinsic growth patterns.

Important as the hygroscopic nature of acid mucopolysaccharide may be mechanically, other explanations for its presence in the stellate reticulum must be considered.

A consideration of the hypotheses put forward by Scott (1968) on the properties of polycations (e.g. polysaccharides) and polyanions (e.g. proteins), leads to the view that their presence in the stellate reticulum could account indirectly for the sudden increase in intercellular material there, its diminution at the time of matrix formation and its ability to furnish ions needed for hard tissue.

Scott's explanation runs as follows:- Polyanions - chiefly proteins which are present in solution along with the acid mucopolysaccharides - have a binding effect on cations such as Na^{+}, H^{+}, K^{+}, which are thus no longer able to exert their osmotic effect. If, however, the parent polyanion becomes associated with a polycation(such as acid mucopolysaccharide), the bound cations are once more released and become osmotically active. Dissociation of the complex as happens in the 'correct' electrolytic concentration again frees the polyanions. The 'swelling pressure' of the solution can thus be considered as controlled by the degree of association of the polycation - polyanion complex. Liberation of the bound ions would give an increase in intercellular material.

Calcium ions are known to have a strong affinity for both the carboxyl and sulphate-bearing colloids, and therefore it would not be inconceivable that calcium may exist either in bound or free form in the stellate reticulum. Further, Scott states that although experimental/

experimental proof is lacking, hypothetically "the complex formation of polycation with polyanion could release sufficient bound ions to give a secondary inorganic precipitate - for example Ca^{++} with HPO_4^{--}".

Such a versatile system suggests to me a more plausible account for the developmental changes seen in the stellate reticulum, than those attributed to its mechanical properties. Proof of its validity must, once again, await experimental investigation.

Comparison of tooth development in the EZL strain and other strains of mouse, shows no major and few minor differences in histological or histochemical detail. The results from the EZL strain would therefore seem to form a suitably valid basis for the comparison of *in vivo* development, and the *in vitro* development to be described in Part II.

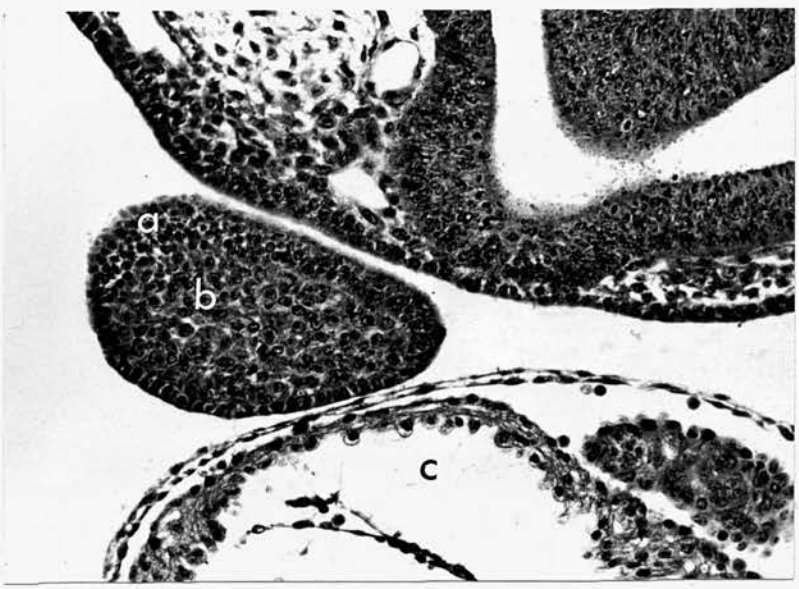
SUMMARY

Normal tooth development in the EZL strain of mouse has been described, and compared with tooth development in other strains as reported in the literature. Special attention was paid to histological details, and to the distribution of glycogen, neutral and acid mucopolysaccharide and collagen. Less detailed descriptions were given of the development of Meckel's cartilage, the dentary, submandibular and major sublingual glands, and the thyroid gland.

The roles of the blood supply, the basement membrane, the mesenchymal condensation and neural crest cells were discussed in the light of interpretations reported in the literature, of their function in the induction of tooth formation.

The significance of the presence and distribution of the histochemical features was also discussed.

FIGURES 5 - 46.



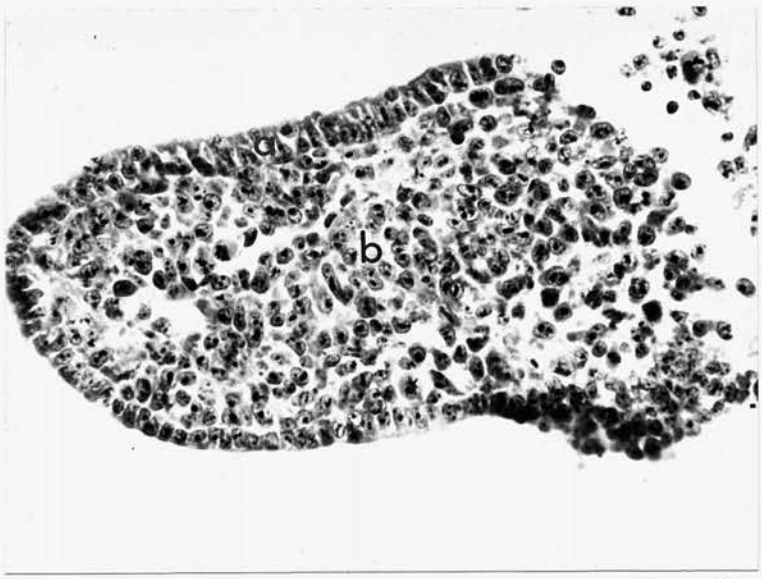
Allochrome x 62.

Fig. 5. Mandible 8 day embryo.
coronal section.

a) 'oral' epithelium.

b) undifferentiated mesenchyme.

c) developing heart.

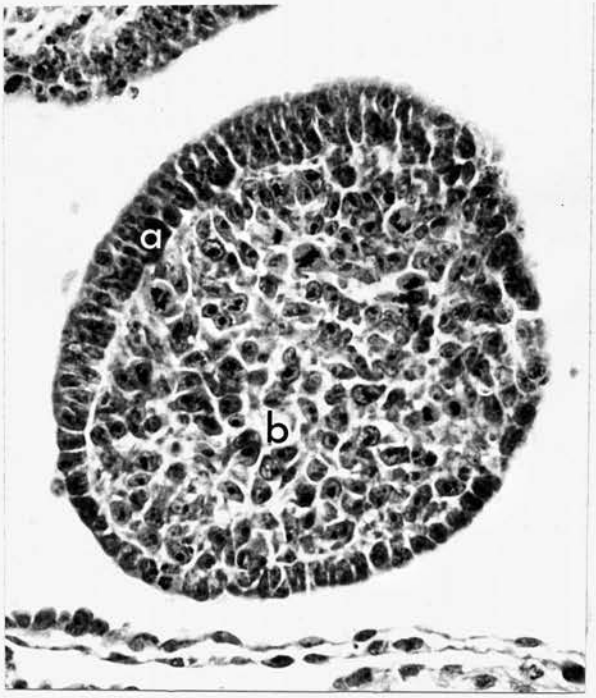


a) 'oral' epithelium.

b) undifferentiated mesenchyme.

H. & E. x63.

Fig. 6. Mandible 9 day embryo. sagittal section.



H. & E. x63.

Fig. 7. Mandible 9 day embryo. coronal section.

a) 'oral' epithelium.

b) undifferentiated mesenchyme.

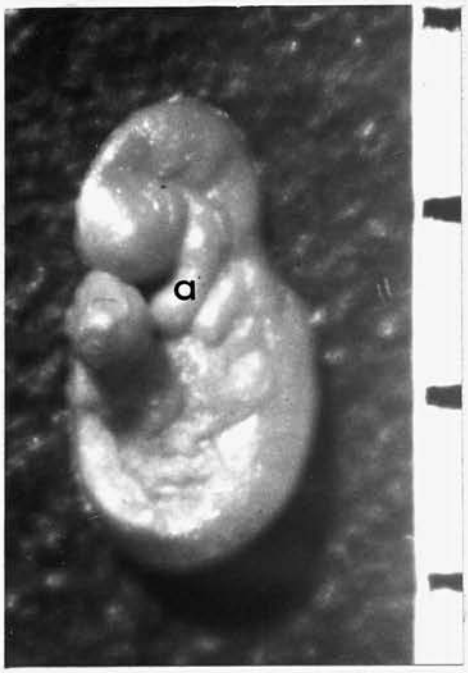
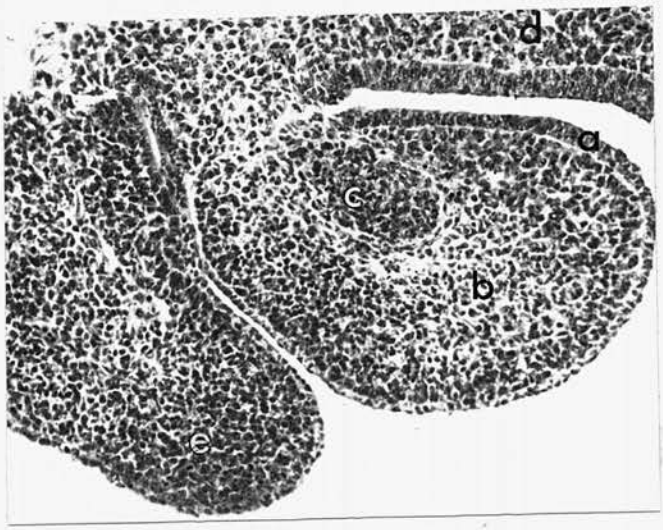


Fig. 8. 9 day embryo.

a) mandible.

scale - mm.



a) 'oral' epithelium.

b) undifferentiated mesenchyme.

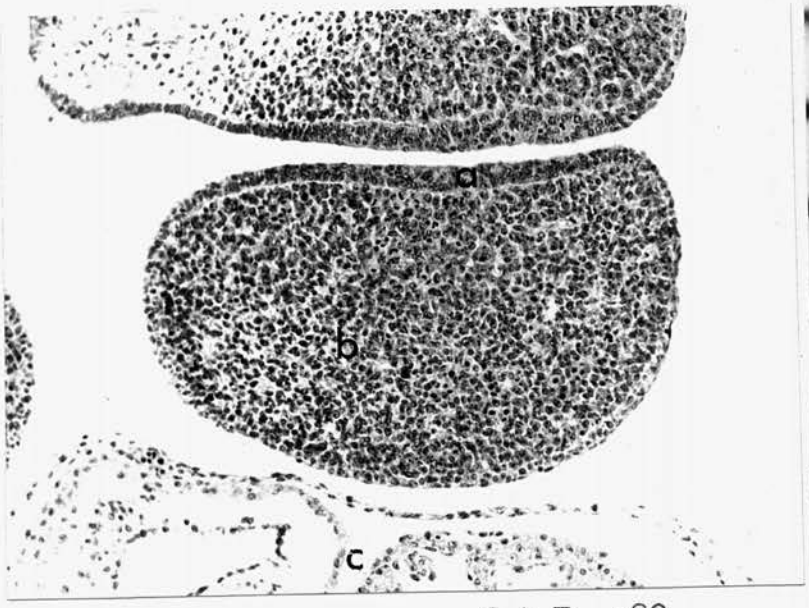
c) primordium of Meckel's cartilage

d) forebrain.

e) hyoid arch.

H. & E. x80.

Fig. 9. Mandible IO day embryo. sagittal section.



H. & E. x80.

Fig. Io. Mandible IO day embryo. coronal section.

a) 'oral' epithelium.

b) mesenchyme.

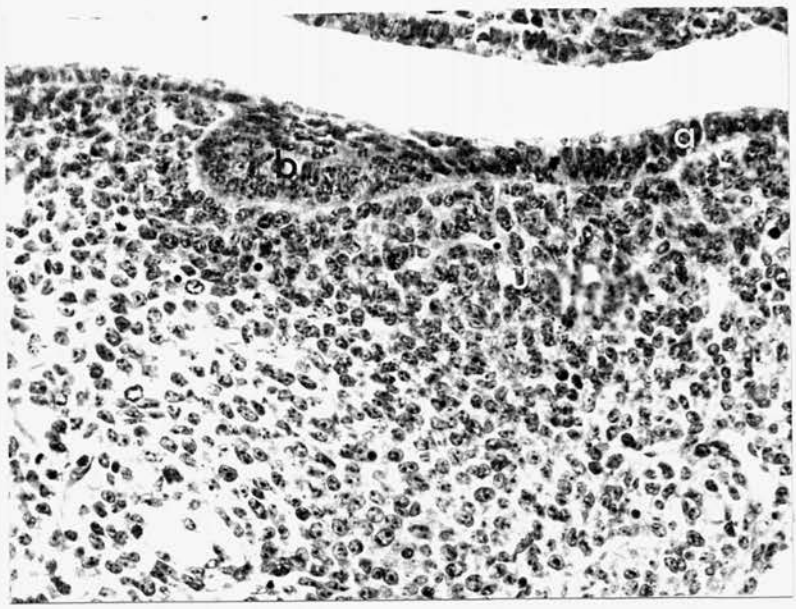
c) developing heart.

Fig. II. IO day embryo. scale - mm.

a) maxillary process.

b) mandibular arch.

c) hyoid arch.

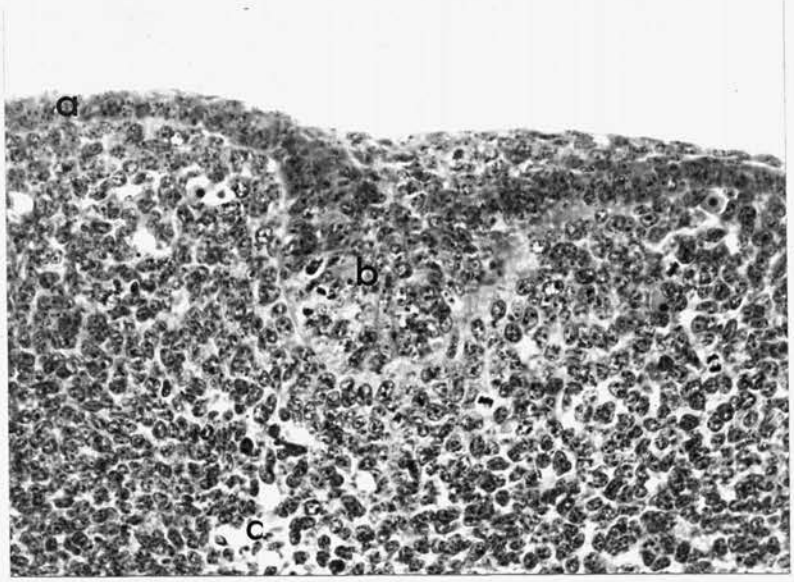


H. & E. x80.

Fig. I2. Mandible II day embryo. coronal section.

a) oral epithelium.

b) dental lamina.



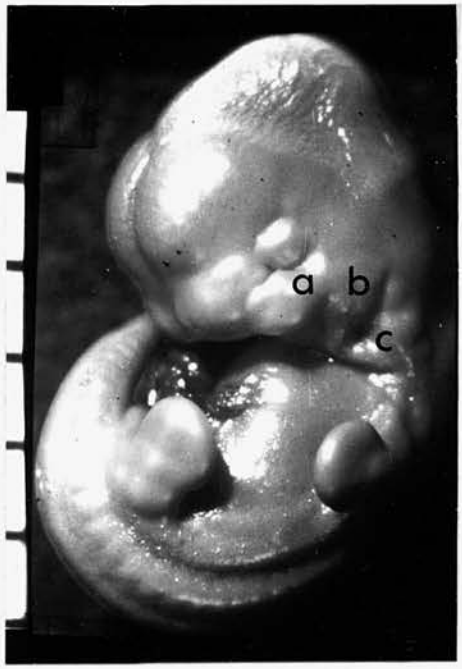
a) oral epithelium.

b) dental lamina.

c) mesenchyme.

Allochrome and diastase x200.

Fig. I3. Mandible I2 day embryo. coronal section.



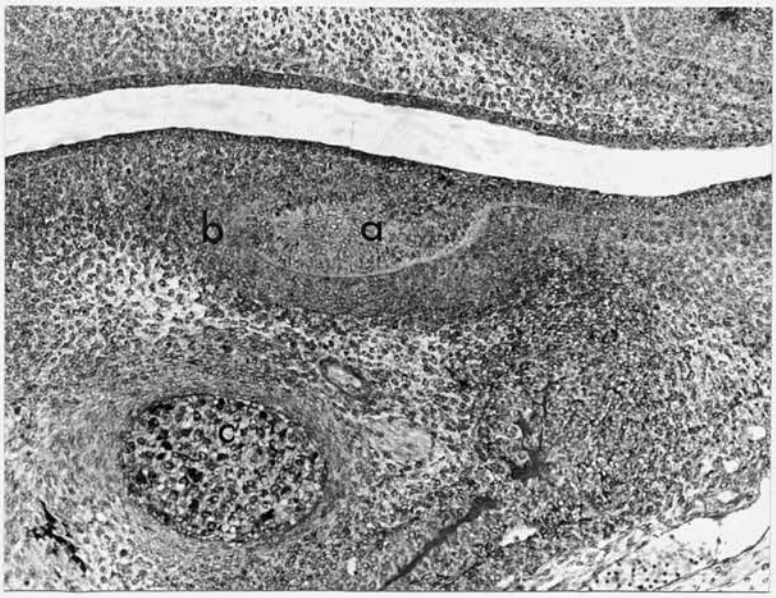
a) maxillary process.

b) mandibular arch.

c) hyoid arch.

Fig. I4. I2 day embryo.

scale - mm.



a) dental lamina.

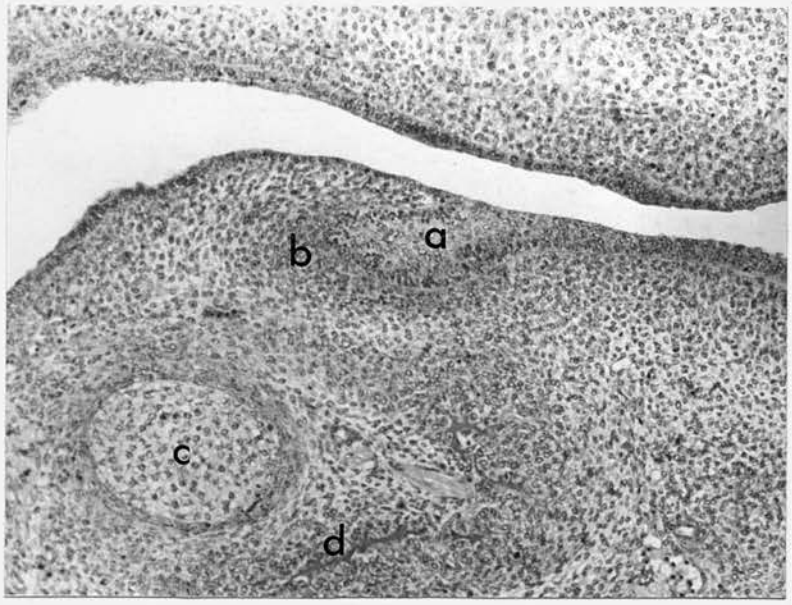
b) mesenchymal condensation.

c) Meckel's cartilage

d) dentary.

Allochrome & diastase x40.

Fig. I5. Incisor I3 day embryo. sagittal section.



Allochrome x40.

Fig. I6. Molar I3 day embryo. sagittal section.

a) dental lamina.

b) mesenchymal condensation.

c) Meckel's cartilage.

Fig. I7. I3 day embryo.

scale - mm.

H. & E. x80.

Fig. I8. Incisor I4 day embryo. sagittal section.

a) oral epithelium.

b) lip furrow band.

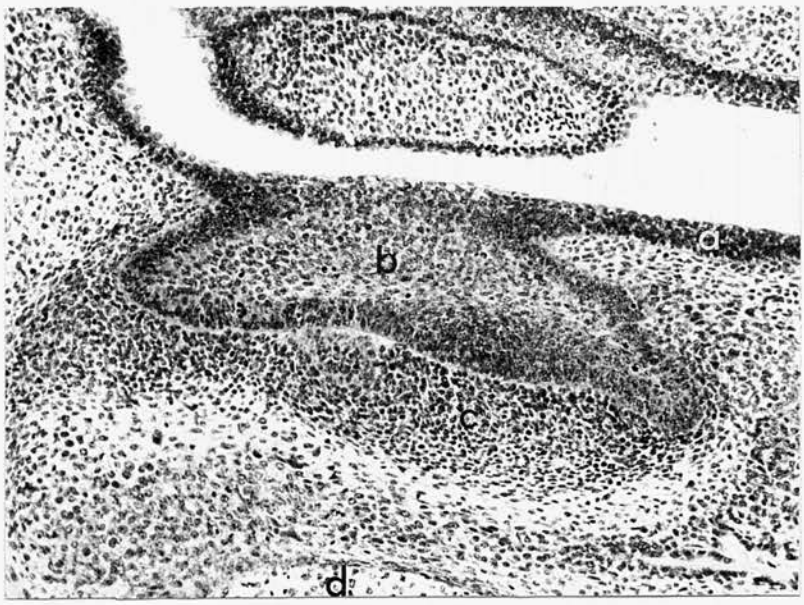
c) enamel organ.

d) dental papilla.

e) nucleated R. B. Cs..

f) Meckel's cartilage.

Fig. I9. I4 day embryo. Scale - mm.



a) oral epithelium.

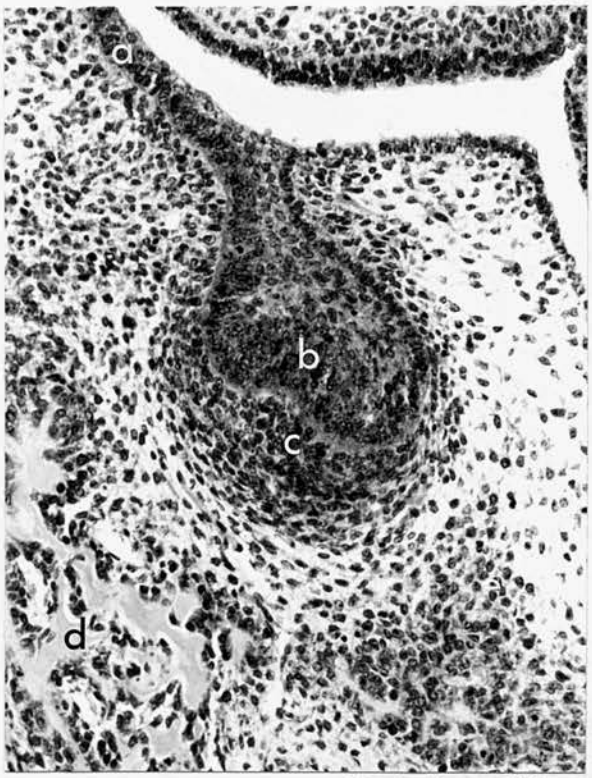
b) enamel organ.

c) mesenchymal condensation.

d) Meckel's cartilage.

H. & E. x80.

Fig. 20. M_1. I4 day embryo. sagittal section.



a) oral epithelium.

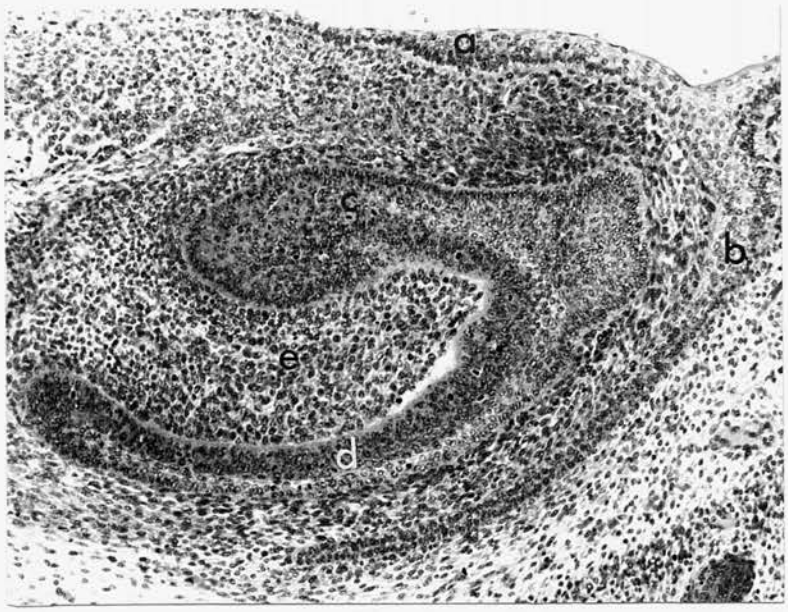
b) enamel organ.

c) mesenchymal condensation.

d) dentary.

H. & E. x80.

Fig. 2I. M_1. I4 day mandible. coronal section.



H. & E. x80.

Fig. 22. Incisor, 15 day embryo. sagittal section.

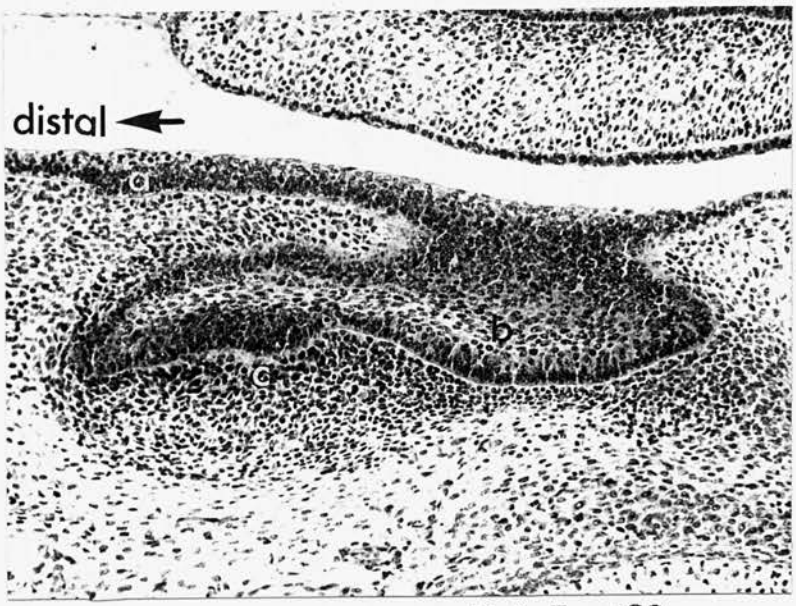
a) oral epithelium.

b) lip furrow band.

c) enamel organ - lingual wall.

d) enamel organ - labial wall.

e) dental papilla.



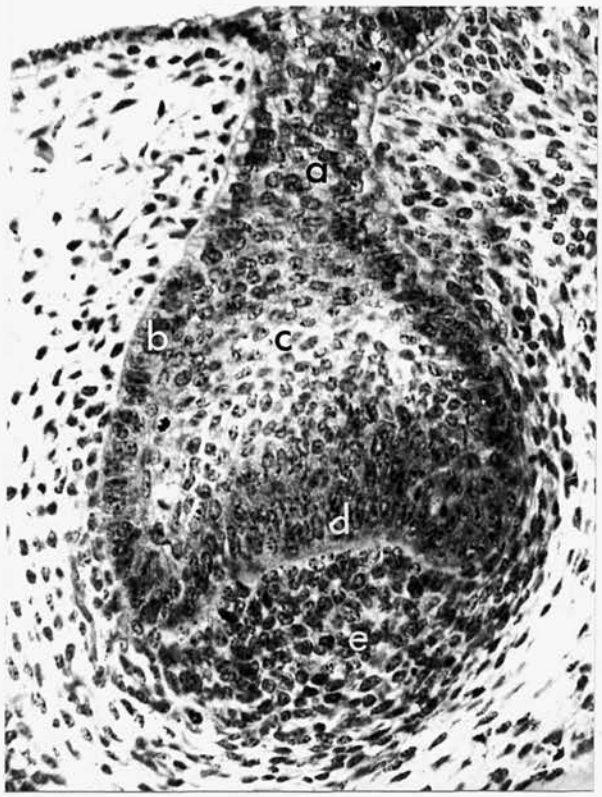
a) oral epithelium.

b) enamel organ.

c) mesenchymal condensation.

H. & E. x80.

Fig. 23. M_1. I5 day embryo. sagittal section.



a) dental lamina.

b) external enamel epithelium.

c) developing stellate reticulum.

d) internal enamel epithelium.

e) mesenchymal condensation.

H. & E. x200.

Fig. 24. M_1. I5 day embryo. coronal section.

H. & E. x 80.

Fig. 25. Incisor, I6 day embryo. coronal section.

a) oral epithelium.

b) lip furrow band.

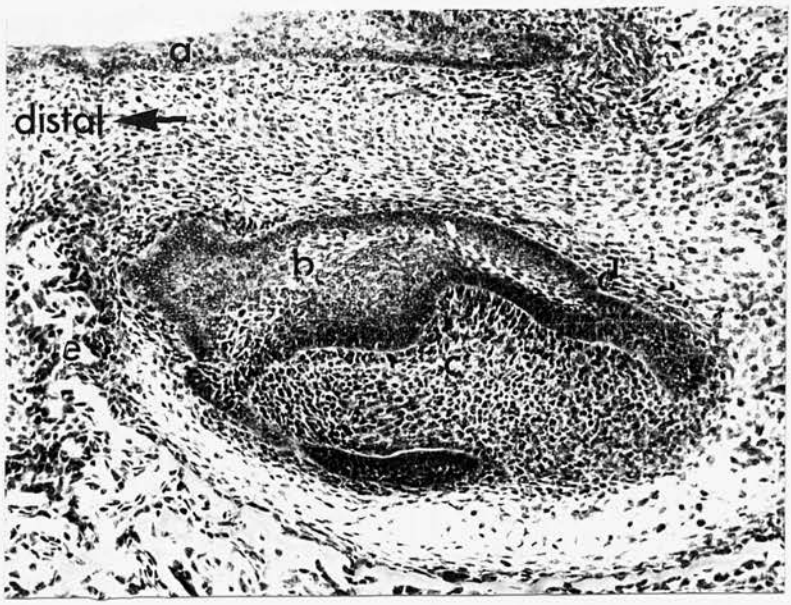
c) internal enamel epithelium.

d) dental papilla.

e) enamel organ.

f) Meckel's cartilage.

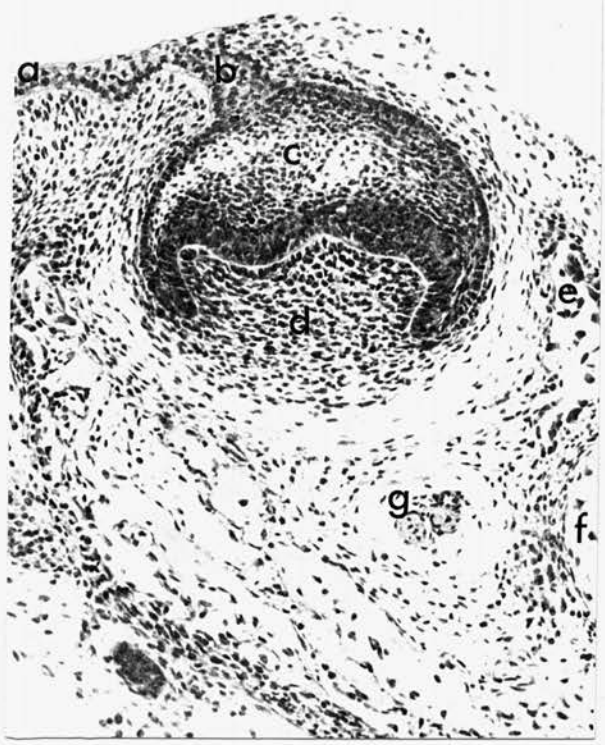
g) dentary.



a) oral epithelium.
b) enamel organ.
c) dental papilla.
d) dental follicle.
e) alveolar bone.

H. & E. x80.

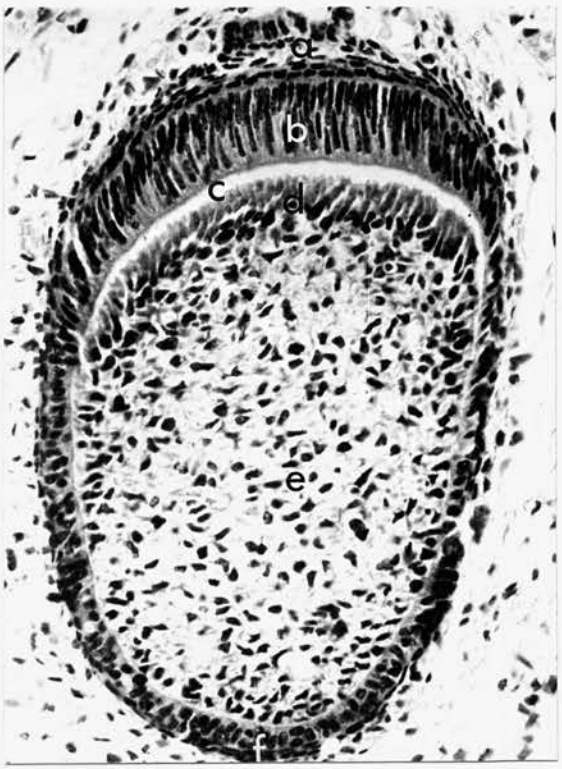
Fig. 26. M_1. I6 day embryo. sagittal section.



a) oral epithelium.
b) dental lamina.
c) enamel organ.
d) dental papilla.
e) alveolar process of dentary.
f) Meckel's cartilage
g) mandibular nerve.

H. & E. x80.

Fig. 27. M_1. I6 day embryo. coronal section.



H. & E. x200.

Fig. 28. Incisor I7 day embryo. coronal section.

a) enamel organ on labial wall of tooth.

b) preameloblasts.

c) predentine matrix.

d) odontoblasts.

e) dental papilla.

a) tongue.

b) dental lamina.

c) stellate reticulum.

d) internal enamel epithelium.

e) dental papilla.

f) alveolar bone.

H. & E. x50.

Fig. 29. M_1. 17 day embryo coronal section.

a) oral epithelium of cheek.

b) M_1.

c) cusp B_1

d) cusp B_2

e) cusp B_3

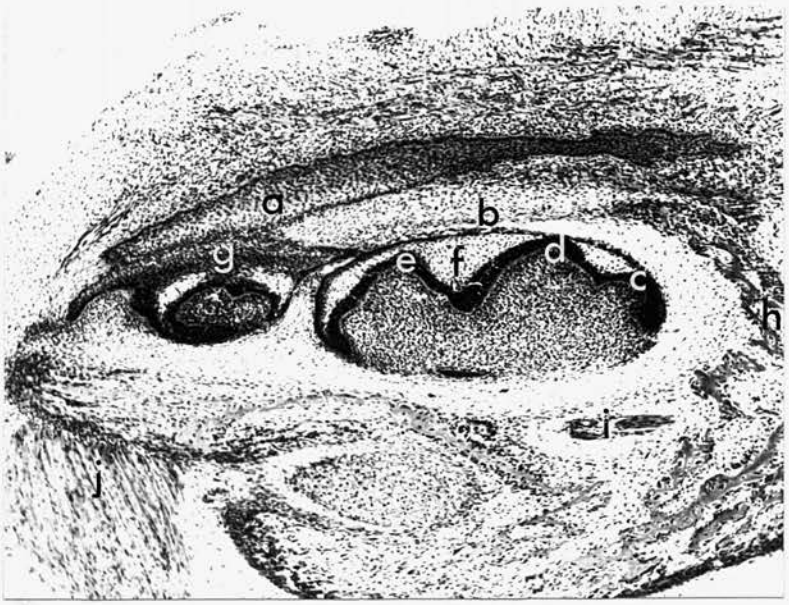
f) stellate reticulum.

g) M_2

h) alveolar bone.

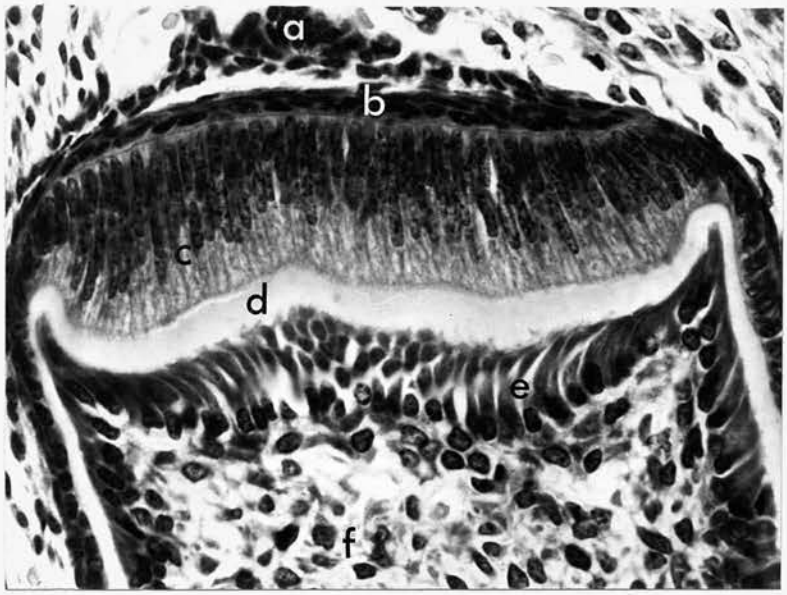
i) mandibular nerve.

j) muscle.



H. & E. x32.

Fig. 30. M_1 and M_2 17 day embryo. sagittal section.



H.& E. x320.

Fig. 3I. Incisor labial wall, I8 day embryo. coronal section.

a) stellate reticulum.

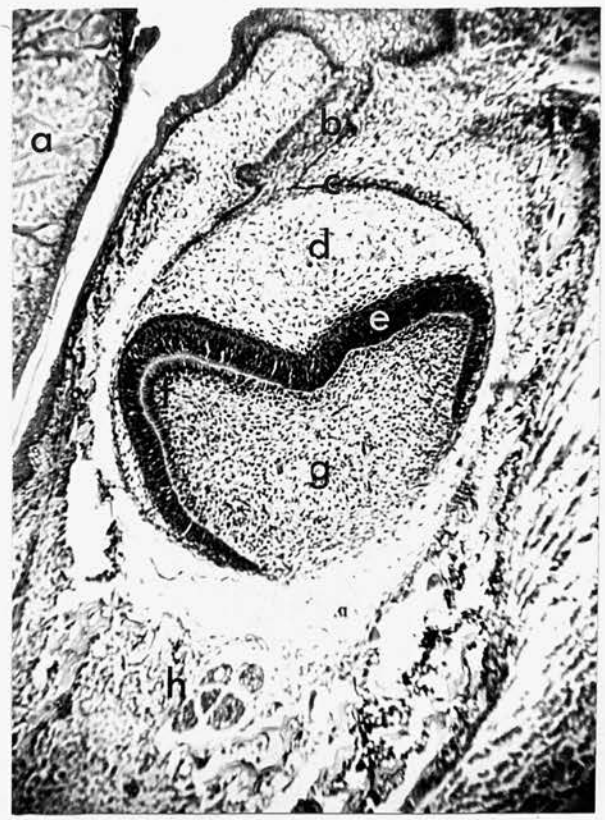
b) stratum intermedium.

c) preameloblasts.

d) predentine.

e) odontoblasts.

f) dental papilla.



H. & E. x80.

Fig. 32. M_1. I8 day embryo. coronal section.

a) tongue.

b) dental lamina.

c) external enamel epithelium.

d) stellate reticulum.

e) internal enamel epithelium.

f) odontoblasts - differentiating on lingual cusp.

g) dental papilla.

h) mandibular nerve.

H. & E. x80.

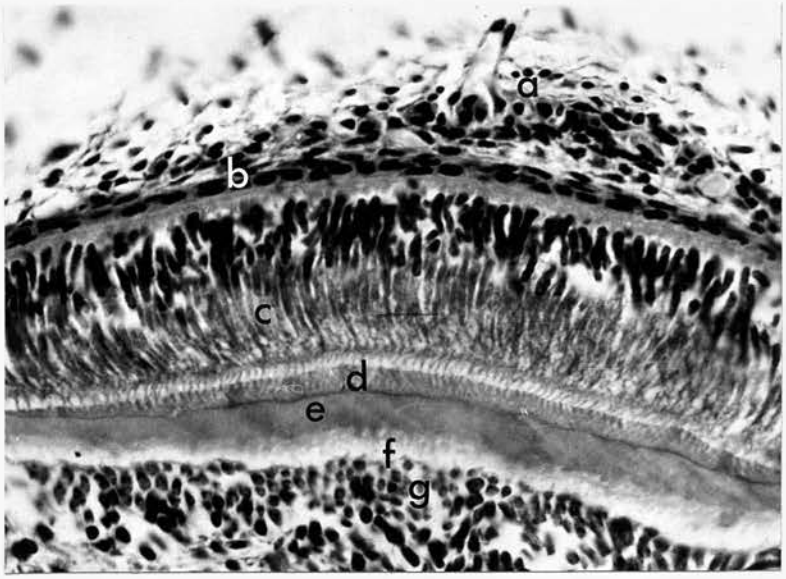
Fig. 33. M_2. I8 day embryo. coronal section.

a) dental lamina.

b) developing stellate reticulum.

c) internal enamel epithelium.

d) dental papilla.



H. & E. x320.

Fig. 34. Incisor, labial wall at birth, coronal section

a) enamel organ.

b) stratum intermedium.

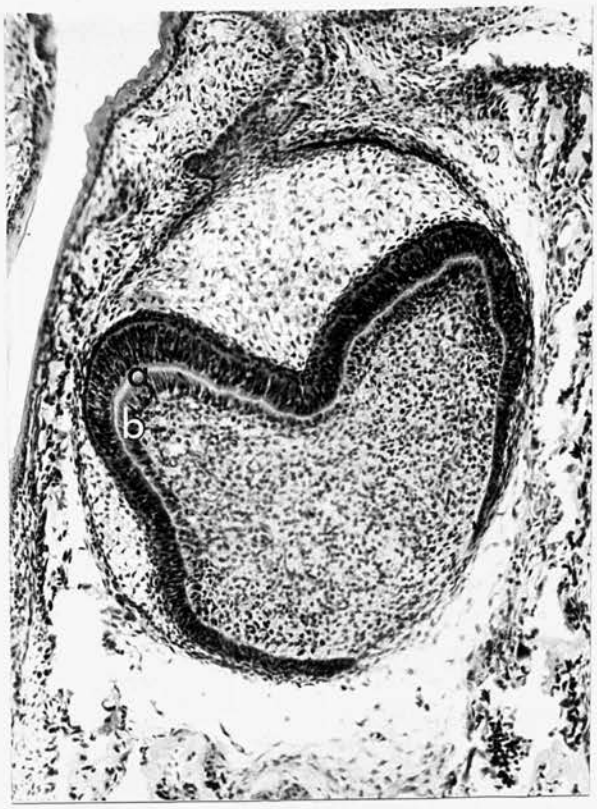
c) ameloblasts.

d) enamel matrix.

e) maturing dentine.

f) predentine.

g) odontoblasts.



H. & E. x80.

Fig. 35. M_1. At birth. coronal section.

As in Fig. 28

but

a) predentine.

b) odotoblasts.

H. & E. x80.

Fig. 36. M_2. At birth. coronal section.

a) dental lamina.

b) stellate reticulum

c) internal enamel epithelium.

a) tongue.

b) dental lamina.

c) lingual cusp more fully developed then buccal cusp.

d) cervical loop region of incisor.

e) mandibular nerve.

f) muscle.

H. & E. x32.

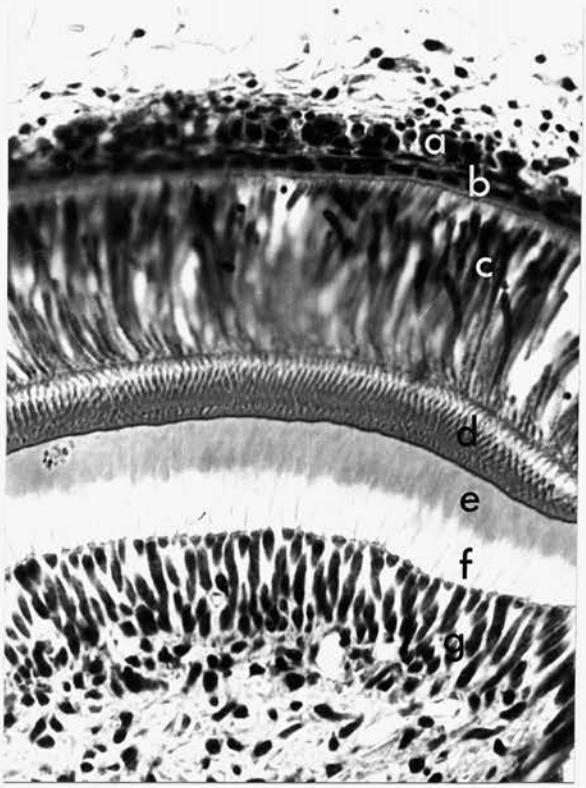
Fig. 37. Mandible - I day mouse. coronal section.

a) odontoblasts differentiating.

b) capillaries invading enamel organ.

H. & E. x80.

Fig. 38. M_2. I day mouse. coronal section.



a) external enamel epithelium.

b) stratum intermedium.

c) ameloblasts.

d) prismatic enamel matrix.

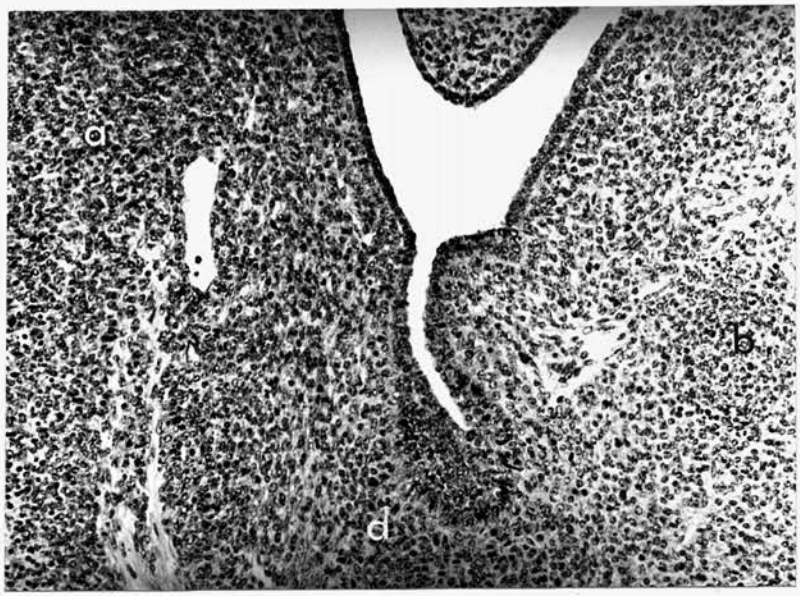
e) maturing dentine.

f) predentine.

g) odontoblasts.

H. & E. x320.

Fig. 39. Labial wall of incisor. 3 day mouse.



a) tongue.

b) mandibular arch.

c) palatal process.

d) mesenchymal condensation - salivary gland primordium.

H. & E. xI25.

Fig. 40. I2 day embryo - primordium of submandibular salivary gland.

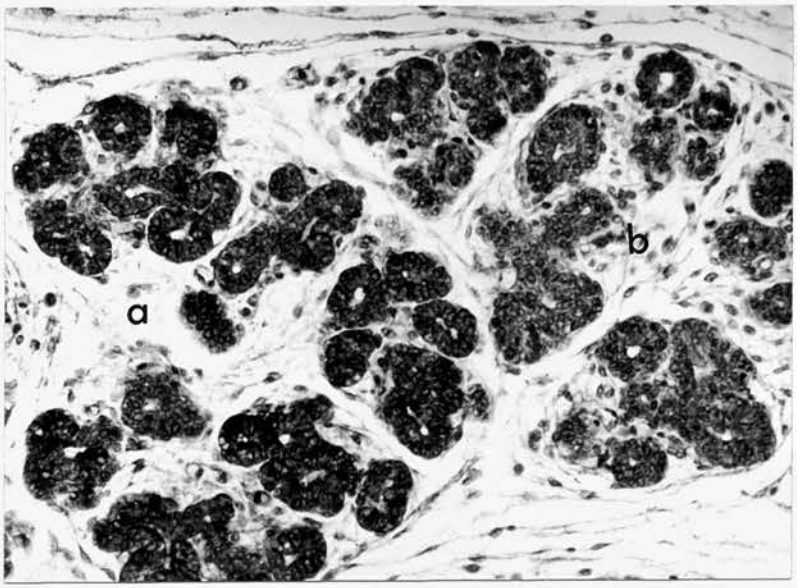
a) tongue.

b) invaginating epithelium.

c) mesenchymal capsule.

H. & E. xI00.

Fig. 4I. I4 day embryo - submandibular gland and duct development.



Allochrome xI60.

Fig. 42. I7 day embryo, submandibular and major sublingual gland.

a) submandibular gland ducts and acini.

b) sublingual gland ducts and acini.

a) oral epithelium.

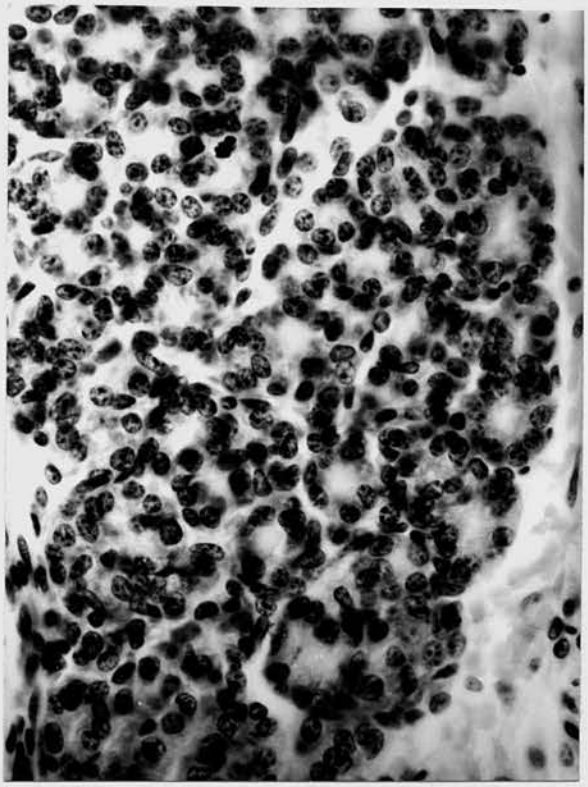
b) mesenchyme of tongue.

c) epithelial primordium of thyroid gland.

d) developing heart.

H. & E. x100.

Fig. 43. Mandible - 9 day embryo to show primordium of thyroid gland.



H. & E. x400.

Fig. 44. Thyroid gland - 17 day embryo to show vesicle formation.

Fig. 45.

DEVELOPMENT OF ODONTOGENIC MANDIBULAR TISSUE - IN VIVO.

x : glycogen.

o : neutral mucopolysaccharide.

• : acid mucopolysaccharide.

DEVELOPMENT OF ODONTOGENIC MANDIBULAR TISSUE - IN VIVO

DISTRIBUTION OF GLYCOGEN, ACID AND NEUTRAL-MUCOPOLYSACCHARIDES

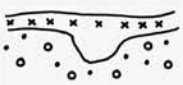
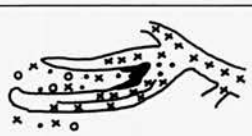
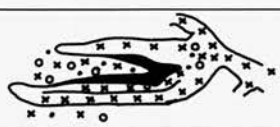
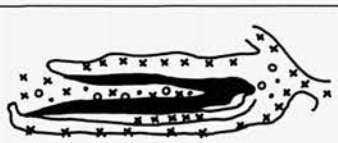
AGE IN DAYS	ODONTOGENIC ORAL EPITHELIUM	MOLAR TOOTH GERM	INCISOR TOOTH GERM
8D			
9D			
10D			
11D			
12D			
13D			
14D			
15D			
16D			
17D			
18D			
19D BIRTH			
1D AFTER BIRTH			

Fig. 45.

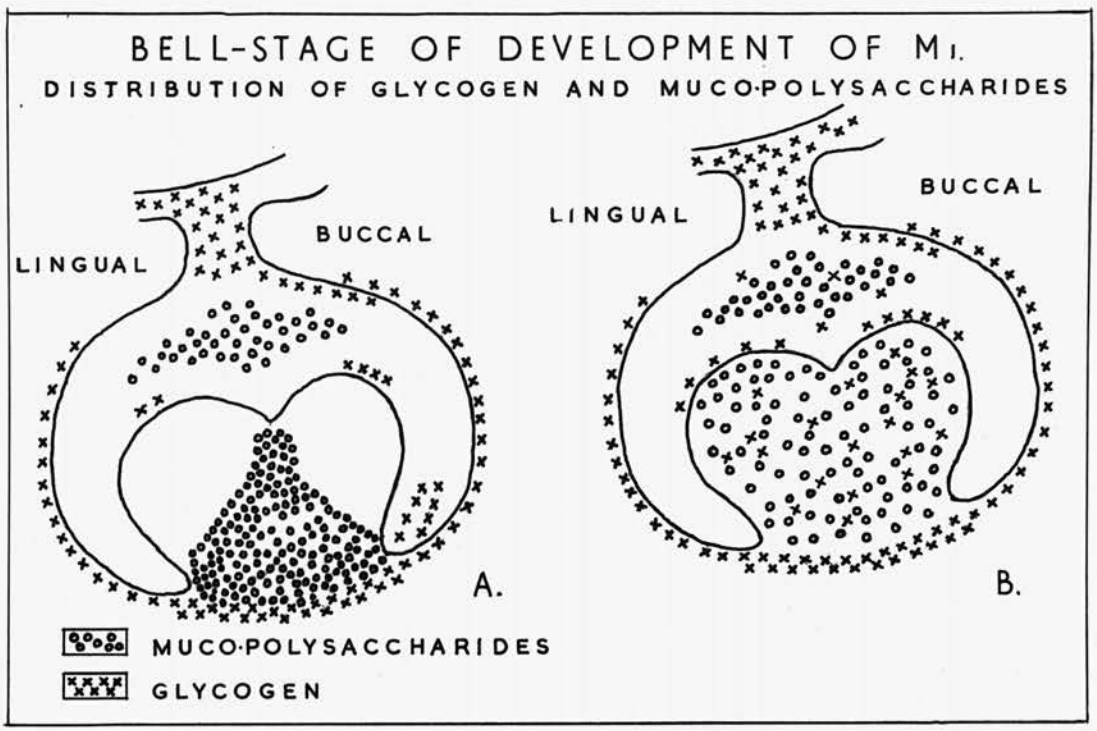


FIG. A. DISTRIBUTION OF GLYCOGEN AND MUCO-POLYSACCHARIDES ADAPTED FROM POURTOIS (1961)

FIG. B. DISTRIBUTION OF GLYCOGEN AND MUCO-POLYSACCARIDES IN THE EZL STRAIN OF MOUSE.

Fig. 46.

PART II.

DEVELOPMENTAL POTENCIES *IN VITRO* OF ODONTOGENIC MANDIBULAR TISSUES.

INTRODUCTION

Organogenesis has been defined by Weiss (1939) as "the series of changes culminating in the establishment of complex, structurally perfect and fully functional organs. The morphological transformations accompanying it are called morphogenesis, and the internal modifications affecting the tissues are called histogenesis".

Morphogenesis and histogenesis, as they are concerned in the organogenesis of the tooth, have been described in detail in Part I. Histogenesis, however, lags behind another developmental change in the tissues, one which, unlike the other changes, can not be investigated microscopically : it is called 'determination' and is the subject of Part II of the thesis.

Tissues, once determined, no longer have the indefinite future of the pluripotent cells of the early embryo, but have a highly specific fate. Determination of the cells - which is fundamentally a change in the active genetic control of their future - is measured experimentally by the capacity of the cells to be self-differentiating under conditions which interfere with their normal in vivo environment. To find out when odontogenic tissues become determined for tooth formation, the procedure of choice is that of isolating the tissues concerned at known stages of development, and of culturing the fragments (explants) in organ culture. Under tissue culture conditions, cells may undergo modulation and appear to de-differentiate, although the retention of their determined state is apparent when they continue organogenesis on being transferred to a suitable milieu, e.g. on transplantation. Organ culture, on the other hand, is designed to prevent modulation and to give conditions which encourage both morpho- and histo-differentiation, thus allowing the tissues to express such properties of self-differentiation as they possessed at/

at the time of explantation.

Under organ culture conditions, isolated fragments or whole arches of the mandible have been encouraged to express any capacity for self-differentiation that they may have had. Each day of age in the mouse from the first appearance of the arch, until birth, has been examined:- 8½-12 days by Pourtois (1964); 9-12 days (1956) and 13-18 days (1953, 1961), by Hay: 11 days by Glasstone (1963, 1965, 1967); 13-19 days by Fisher (1957); 13-15 days by Kollar & Baird (1968); 16 days by Koch (1965) and 17 days by Glasstone (1939). It was found that all tissues explanted after 12 days gestation age and cultured, were self-differentiating to the extent that stellate reticulum, ameloblasts, odontoblasts and predentine differentiated in a tooth identifiable from its morphological features as an incisor or a molar tooth germ.

Fully differentiated tooth germs have never been observed in vitro. There is little evidence of calcification of the dentine matrix (Koch, 1967) and although enamel has been found on several occasions, it is by no means a constant feature of cultures whose morpho- and histo-differentiated states would lead one to expect its presence. Glasstone (1964) found it in 10% of incisor tooth germs from such cultures. Hay (1961) found it in 23% of them, and Koch (1965) almost invariably. All authors noted that any enamel present was scant and abnormal in structure, usually with a fibrous appearance.

Slight abnormalities have also been noted in morpho-differentiation of cusps in cultured tooth germs. Fisher (1957) found that cultured molars from 13-14 day mouse embryos often showed fusion of cusps B_1 and B_2, and of cusp 4 with L_3 and B_3.

There is general agreement that these abnormalities in development in culture are due to the inadequacy of the culture method to mimic in vivo conditions, and not to a lack of determination of the explanted tissues.

It/

It is of interest to note that work done on equivalent stages of development in rat tooth germs illustrate the same finding that, once the dental lamina is well established in the mandible, the tissues are determined for tooth formation. Such work has been done by Glasstone (1936, 1939); Lefkowitz, Mardfin & Bodecker (1954); Szabo (1954); Gerstner & Butcher (1958) and Lefkowitz & Swayne (1954, 1956a, 1956b, 1958).

Culture of mouse dental tissues from the period of 12-days gestation age to birth has thrown light on other aspects of determination. Determination is not considered to take place abruptly, but to procede by a series of events, directing future development more and more precisely (Waddington, 1962). Many experiments illustrate the 'plasticity' of the early determined stages of teeth, similarly to other organs. Glasstone (1952) found that tooth germs of the rabbit, on being halved transversely at the 20-21 days gestation stage, were capable of abandoning their apparently determined path of development, and reorganising themselves into two small tooth germs with normal morphological features. After matrix formation commenced, regulation ceased.

Fisher (1957) repeated such experiments with mouse tissues, and found that tooth germs halved at the 13-14 day stage (just before the cap stage of development) were able to regulate themselves, and one of the halves in each case developed into a small typical tooth germ in vitro. All later stages were incapable of regulation, and seemed therefore to be fully determined. Another discovery made by Fisher (1957), but one which so far has not been verified, was that on culturing left-hand-side mandibles of 13-14 day embryonic mice, 55% of cases developed molars with a right-hand-side configuration as checked by wax models. She attributed the apparent abnormality of development in culture to the plasticity of the tissue at the time of explantation.

Once odontogenic tissue is determined, it can retain its ability to be self-differentiating over a long period of time, as was/

was found by Main (1966). Emrbyonic mouse tissues explanted at the 14-day stage and cultured in gelatin sponges, were found to modulate under the conditions of growth, and remained viable for 37 days. On transplantation into isologous newborn mice, they formed well developed incisors with normal, pigmented enamel - thus demonstrating their innate determined state at the time of explantation.

The conclusions drawn from all these reports are, a) that, by the time the dental lamina is invaginating in the mandible, the tissues are already determined for tooth formation; b) that, for some time, they are not in a fully determined state but exhibit plasticity, and c) that they retain their determined state on modulation in tissue culture conditions. The observations do not, however, define an initial time of determination.

The observations discussed above, indicate that initial determination of mouse tooth germs must take place before the 12-day stage, and, by analogy with other organs, it would be expected to take place before the first sign of histodifferentiation of the dental lamina is seen, i.e. before the 11-day stage. Attempts have been made to find this initial stage.

Glasstone (1963; 1965) found that fragments isolated from 11-day embryonic mouse jaws developed well in culture to form M_1 and M_2. Hay (1956) found also that this was the youngest age at which mandibular fragments would develop <u>in vitro</u> to form recognisable tooth germs. Incisor tooth germs appeared normal, but of the two molar tooth germs that developed, one showed cusp formation but no differentiation of the stellate reticulum or stratum intermedium expected at this stage, and in the other, odontoblasts had developed, but no predentine was present. Pourtois (1964) found no recognisable tooth germs in cultures from this stage. Under optimum culture conditions, however, it would seem that a capacity for self-differentiation could be elicited from this stage of development.

Hay/

Hay (1956) and Pourtois (1964) also examined mandibular tissues from the 10-day old embryo where epithelium and mesenchyme are as yet undifferentiated. All fragments of jaws isolated and cultured by Hay degenerated, but in one case, where a whole arch was cultured, a molar tooth germ developed showing early cusp formation and pre-ameloblast differentiation. Similar experiments by Pourtois, yielded tissues that could have resembled abnormal enamel organs, and a type of dentigerous cyst, but no recognisable tooth germ. Pourtois attributed lack of differentiation in vitro to the trauma of dissection, and Hay to inadequate culture conditions.

Both Hay (1956) and Pourtois (1964) found that isolated mandibles from 9-day embryos, on culturing, formed no recognisable tooth germs.

Pourtois (1964) alone, cultured explants from 8½-day embryo mandibles, and except for one explant, all degenerated. In the surviving explant, cuboidal epithelium and undifferentiated mesenchyme were found.

Grobstein (1951) cultured complete embryonic shields of 7-day mice in vitro for two days, and then in the anterior chamber of the eye of the mouse host. After 28 days, one implant was found to contain a tooth with a thick layer of dentine and enamel. However, as the complete embryonic shield in this case was cultured, the experiment does not throw much light on the specific organogenesis of the tooth.

Conclusions drawn from these reports of in vitro development of mouse mandibles isolated at 8½-12 days gestation age are, a) that at 11 days the tissues are determined to some extent for tooth formation; b) that the one example of a tooth developing from a 10-day mandible suggests that under ideal culture conditions the tissues would be found to be determined, and c) the widespread degeneration in culture of tissues explanted from 8- and 9-day mandibles prevent us from drawing conclusions about their developmental/

developmental state. All results seem to indicate that tissues explanted from very early embryonic stages are much more exacting in the conditions under which they will thrive in vitro than older explants.

The experiments I have carried out, and report here, were with mandibular tissues of mice of 8-14 days gestation age. The experiments made with tissues from 11-14 days gestation age were to verify that such explants from the EZL strain of mouse were self-differentiating in culture, and to find out some details of their cytochemical, as well as their morphological and histological development in vitro. The experiments made with tissues from 8-11 days gestation age were designed to find out:-

1) if mandibular tissues from these ages would differentiate well in vitro,
2) if development takes place - how closely does it resemble in vivo development,
3) if tooth germs develop - at what stage do the mandibular tissues appear to be determined for tooth formation?

METHODS AND MATERIALS

All tissue culture terms used are as proposed by Federoff (1967).

1) Preparation of the explants

Throughout the experiments, from the removal of the gravid uterus to fixation of the explants, all apparatus and media used were sterile, and all procedures were carried out aseptically.

Pregnant females of the EZL strain of mouse were killed by cervical dislocation, and the uterine horns dissected out intact and placed in Tyrode's solution. Individual embryos were then transferred to horse serum : Tyrode's solution (1:20 $^{V}/_{V}$). The age of the embryos was originally assessed by the appearance in the mother of the vaginal plug, but at this stage, was confirmed by observation of the embryos' external features. Any retarded embryos were discarded, and one litter mate was immediately fixed as a control for each experiment. The mandibles of the remaining embryos were dissected out and, according to the age of the embryo, were further divided; in older embryos, where odontogenic areas could be identified by the epithelial thickenings or by the localised increased blood supply, the incisors and molars were isolated; in younger mandibles, the ventral presumptive epithelium was removed, the mandible divided in the mid-line, and each arch either explanted whole, or divided into an anterior and posterior half. In very young mandibles, the arches were not divided at the mid-line. Dissection was carried out under a binocular dissecting microscope, using sharpened cataract knives and stainless steel needles. Tissues were washed in Tyrode's solution, then explanted.

2) Methods of cultivation.

a)/

a) The Watch-glass technique (Fell & Robinson, 1929) was the method of choice for the older explants (9-14 days). The culture medium used was plasma : embryo extract ($5:2^{V}/_{V}$).

b) Modifications of the hanging - drop method were used for 8-day and the smaller 9-day explants. The double coverslip method of Maximow (see Parker, 1961) was used for the youngest explants at early stages of culture. After initial growth of the explants they were either transferred to watch glasses, or occasionally the method of Losee (1943) was used, in which the plasma clot is held in the depression of the hollow-ground slide, and not on the coverslip. The clots used in these methods were composed of plasma: embryo extract ($2:1^{V}/_{V}$). Incubation in all cases was at $37.5^{o}C$.

The media used in the Watch-glass and hanging-drop techniques were:-

a) Tyrode's solution (Paul, 1961).

b) Plasma. Fresh fowl plasma, obtained from the wing vein or carotid artery was used (Paul, 1961). Premature clotting was avoided by the use of chilled, siliconed apparatus. Plasma was stored in the refrigerator for periods of up to one week, then fresh plasma was prepared.

c) Embryo extract. Chick embryos of 10 or 11 days incubation were used. The eye and gall bladder were removed, then the extract was prepared by mincing with scissors (Buchsbaum, 1936). The embryo extract used was composed of the supernatent after the concentrated minced embryo was diluted with twice its volume of Tyrode's solution, and centrifuged at 2,000 r.p.m. for 10 minutes.

d) Antibiotics were not used until during the last few experiments, when roof repairs to the laboratory greatly increased the risk of contamination. Nystatin (Mycostatin, Squibb), in the concentration of 100 units/ml. of medium was used (Parker, 1961). On two occasions sodium penicillin G, at a concentration of 50 units/ml., was also used (Paul, 1961).

3)/

3) Subculture.

a) Watch-glass technique. Explants were subcultured every second day (Fisher, 1957). The explants were dissected from the old plasma clots, most of the outgrowth of cells being removed cleanly (Parker, 1961). The explants were washed in Tyrode's solution, and repositioned on freshly prepared clots.

b) Hanging-drop method. Explants were again subcultured every second day. They were dissected out, discarding cellular outgrowth, then washed and embedded in fresh clots. At the first subculture of the 8-day explants, however, a different technique was employed (McLoughlin, 1959) to discourage the excessive outgrowth of the few cells constituting the explant.

Procedure: The inner coverslip was removed, inverted in Tyrode's solution and allowed to stand for 10-20 minutes. It was then removed and placed on a new outer coverslip. More plasma: embryo extract ($3:2^{V}/_{V}$) was added, and the coverslip sealed over the depression of the slide. At the next subculture, two days later, dissection, washing and transferrence of the explant was as normal.

4) Histological treatment.

Histological treatment was, on the whole, the same as for the control series, outlined in Part I, with a few exceptions.

a) Fixation.)
b) Dehydration.) Were as described in Part I, except that 5% eosin was added to the 98% alcohol. This stained the explants before embedding, and made them more visible for subsequent handling.

c) Embedding. Drawings were made at the time of embedding, of the orientation of the explants within the blocks.

d) Sectioning. As in Part I. No more than six sections of any one explant were placed on one slide, to allow any tooth germ present to be stained as many ways as possible.

e)/

e) Staining. As in Part I. Consecutive slides were stained routinely with Haematoxylin and Eosin, Allochrome, Allochrome and diastase, and the Alcian blue method.

5) Methods of Examination.

a) Living cultures were examined each day under the dissecting microscope. Before subculturing every second day, drawings were made of each one, indicating shape, size and outgrowth of the explant.

b) With the 8 day explants, photomicrography was possible because of the translucency of the tissues. Photographs were taken of the living cultures every second day.

c) Straightforward examination of the histological sections was made.

d) On occasion, to interpret 3-dimensional shape of the tooth germs which developed in culture, camera lucida drawings were made of the tissues from the serial sections prepared.

6) Preliminary experiments.

Experiment 1. Whole mandibular arches from 11- and 12-day embryos were cultured for periods of 6 to 8 days. The oral epithelium in each case was found to be scant, and showed few mitoses. It was thought, therefore, that the culture conditions might be inadequate for healthy growth of the epithelium. Knowing that the energy requirements of epithelium are high, and that sufficient quantities of glucose *in vitro* are essential (Parshley & Simms, 1950; Bullough & Johnson, 1951; Bullough, 1952), the glucose concentration in the medium was examined. It was found to be lower than in normal mouse plasma. Blood glucose level in the mouse is recorded at 174 mg.% - with a maximum of 266 mg.% (Albritton, 1952). Tyrode's solution contains only 100 mg.%, and fowl plasma 200 mg.% (Willmer, 1965) - with the result that the culture media used were theoretically deficient in glucose for the cultivation of mouse tissues. Moscona, Trowell & Willmer (1965), suggested/

suggested that the optimum concentration of glucose in a medium depends not entirely upon the normal plasma level for that animal, but also on the rate of its use in culture - this factor depending on the volume of the medium and the size of the explant. They concluded that the optimum level must be found ad hoc. Glucose was therefore added arbitrarily to the Tyrode's solution - and knowing that cells will tolerate much higher concentrations than they normally have in their surroundings (Moscona et al., 1965) - quite liberally.

Culture of fragments of 14-day embryo mandibles for 12-14 days in a medium where 1% glucose Tyrode's solution was used, resulted in well differentiated tissues, and epithelium showing many mitoses (Figs. 47, 48).

All future experiments were carried out using 1% glucose Tyrode's solution in place of the normal solution.

Experiment 2. When the whole mandibular arches from 11- and 12-day embryos were cultured as described in Experiment 1, not only did the epithelium develop abnormally, but also the mesenchyme. In these large explants, the central cells were found to have degenerated after 6 to 8 days in culture. When the experiments were repeated using 1% glucose Tyrode's solution, the epithelium developed well, but the central areas were still necrotic. Such a finding is common in large explants, and is thought to be due to lack of oxygen, or to a build-up of toxic waste products of metabolism unable to diffuse from the deepest regions of the tissue (Moscona et al., 1965). As embryonic cells obtain most of their energy from glycolysis (Moscona et al., 1965; Bullough, 1952), the most likely toxin in this case is lactic acid. It was decided to raise the oxygen tension experimentally in the atmosphere surrounding the explants. Young embryos are known to be dependent on glycolysis for their energy, but with the establishment of a blood supply, aerobic respiration gradually assumes more importance (Fisher & Schwabe, 1969). Where there is inadequate oxygen, the Pasteur effect/

effect comes into force, and cells use glycolysis for energy production, even though capable of aerobic respiration (Paul, 1965; Papaconstantinou, 1967). It was hoped that glycolysis would be minimised by providing an adequate oxygen tension, and therefore decrease lactic acid production.

The experiments were repeated, with the watch-glasses containing the cultures being stored in the incubator in a dessicator whose contained atmosphere was 58% oxygen and 3% carbon dioxide in air. After 6-8 days culture, the tissues were examined histologically. It was found that the rate of development was unaffected, but that the areas of degeneration were no longer present.

In future experiments, therefore, either a) large explants were divided into smaller fragments which were then subcultured separately, or b) if this treatment was not suitable, the explants were cultured in an atmosphere of approximately 58% oxygen and 3% carbon dioxide in air.

RESULTS

Explants were made from the odontogenic regions of the mandible from successively younger ages of embryos of 14-8 days gestation age, and cultured *in vitro*.

An approximate rate of development of the tooth germ *in vitro* compared to that *in vivo* was calculated along the following lines. When explanted, the development of the tooth germ is slowed. A measure of the retardation is obtained by comparing the number of days of apparent *in vivo* growth with the number of days *in vitro* required to reach the same stage of development, counted in each case from the day of explantation. The resemblance of *in vivo* and *in vitro* tooth germs was assessed on morphological, histological and histochemical features, and the retardation was expressed as a percentage (see Table II).

a) Development of explants from 14-day embryos.

In the 14-day old embryo, the tooth germs are in the cap-stage of development and easily identifiable (Figs. 18, 20, 21), so that individual molar or incisor germs can be isolated and cultured. Six such explants were made. All explants showed continuing differentiation *in vitro*.

An incisor tooth germ cultured for 6 days gave rise to a tooth very similar to that found *in vivo* in a late 16-day embryo - a rate of development approximately 40% of normal. The walls of the tooth germ had elongated appreciably, and the internal and external enamel epithelia were composed of cuboidal cells. In the walls of the enamel organ, the stellate reticulum and the stratum intermedium were much reduced in volume, and a large epithelial pearl had obliterated most of the stellate reticulum at the incisal tip of the tooth. The dental papilla was composed of closely packed mesenchymal cells. Near to the labial surface of the cervical loop region of the enamel organ, osteoid tissue had developed as *in vivo*.

Histochemical/

Histochemical staining revealed that glycogen granules were present in the dental papilla and all cell layers of the enamel organ throughout the length of the tooth, except at the cervical loop region. The dental papilla contained neutral and acid mucopolysaccharides and fine collagen fibrils, less dense than in the mesenchyme surrounding the tooth germ.

After 12 days in culture, three incisor tooth germs which developed in two explants were found to have differentiated to disparate degrees. The least developed tooth germ had reached the stage where predentine was present at the incisal tip, and other histological features of the tooth were entirely in agreement with this in vivo stage. The histochemical appearances were similar to those already described for the incisor of 6 days culture, except that in the older explant, pre-ameloblasts had differentiated in the incisal region, and where the nuclei had moved away from the basement membrane, glycogen granules were seen to have accumulated at the basal (basement membrane) pole of the cell. The granules were not found in the less fully differentiated internal enamel epithelial cells.

Thicker deposits of predentine were found in the other two more elongated incisors. They were otherwise similar to that already described. A higher proportion of the internal enamel epithelium was composed of elongated pre-ameloblasts, and all such cells exhibited the basal glycogen granules. Collagen fibrils were fine at the cervical loop region and were denser and of greater calibre at the incisal tip.

The rate of development of the most fully differentiated tooth germ was at least one-third that of normal, and gave rise to a tooth germ which resembled that of an 18-day old embryo (Fig. 31).

Three molar tooth germs also developed during 12 days in vitro. A different stage of differentiation was achieved by each explant. With the least developed tooth germ, cusps L_1B_1 : L_2B_2 : L_3B_3 were present, but no predentine was deposited (Fig. 47).

Histological/

Histological features were compatible with the stage indicated by the morphological appearances - that of a 16½-17 day embryo (Figs. 26, 28).

Histochemical stains showed glycogen granules in the internal and external enamel epithelia except for the cervical loop region. Finer granules were present in the stellate reticulum and stratum intermedium, and also in the dental papilla. Neutral and acid mucopolysaccharides were present, very sparsely in the stellate reticulum, but more abundantly in the dental papilla. Collagen fibrils were present in the papilla, but less densely than in the surrounding mesenchyme.

With the more fully developed explants, predentine had been deposited, in each case on the 'highest' cusp, identified as a mesio-lingual cusp (Figs. 48 & 68). Glycogen granules were intense in the external enamel epithelium, and some were present in the stellate reticulum and the dental papilla. In these explants, glycogen granules could not be detected in the internal enamel epithelium by special stains, whether the cells were elongated or not. Neutral and acid mucopolysaccharides were present in the stellate reticulum and the dental papilla.

The most fully developed molar tooth germ resembled that found at birth (19 days - Fig. 35) *in vivo* - giving a rate of development *in vitro* of 46% that of normal.

b) Development of explants from 13-day embryos.

In a 13-day old embryonic mouse, separate epithelial invaginations are present in the mandible for both incisor and molar tooth germs (Figs. 15, 16).

Fragments of the mandible, or the oral epithelium and its underlying mesenchyme of the whole arch, were explanted. Twenty-one such explants were made. During culture, recognisable incisor and molar tooth germs developed.

Culture for 2, 4 and 6 days resulted in epithelial invaginations quite consistant with early enamel organ formation - the rate of development being about 50% that of normal.

After/

After 8 days in culture, a more fully differentiated (and therefore more convincingly identifiable) molar tooth germ developed. Cusps L_1B_1 : L_2B_2, and the beginnings of L_3B_3 were recognised. The enamel organ was composed of an internal and external enamel epithelium of cuboidal cells, and the stellate reticulum showed some intercellular spacing. All cell layers of the enamel organ contained glycogen, which was also present in the dental papilla, where the granules were sparser and finer. The papilla also stained positively for the presence of neutral and acid mucopolysaccharides.

The overall picture was consistent with that of a normal tooth germ found in an early 17-day embryo (Fig. 28). The rate of development *in vitro* was therefore about 50% that of normal.

With 14 days in culture, 1 incisor and 3 molar tooth germs could be identified in different explants.

The incisor tooth germ was elongated, one side more so than the other (Fig. 49). The internal enamel epithelial cells of this longer wall were more elongated than elsewhere, and predentine, present for at least two-thirds of the length of the tooth, was thickest on this side - all features identifying it as equivalent to the labial wall *in vivo*. This tooth germ seemed to resemble that of an 18-day embryo (Fig. 31), with a developmental rate *in vitro* of about 36% that of normal.

The 3 molar tooth germs (Fig. 68) were found to have developed to the 18½-19 day stage (Figs. 32, 35), i.e. a developmental rate of about 43% that of normal. Predentine was present in a thin layer at the tip of the highest, most fully developed cusp. The stellate reticulum consisted of much intercellular material, and the cells of the internal enamel epithelium had elongated to form pre-ameloblasts at the tips of the cusps. The histochemical picture was similar to that found in the explants developed from the 14-day *in vivo* stage. No glycogen was found inthe internal enamel epithelium, except in the pre-ameloblasts, but was present in all other cell layers of the enamel organ and in the papilla. The stellate reticulum and dental papilla stained positively for both neutral and acid mucopolysaccharides.

In/

In one larger explant, M_2 had developed as well as M_1, and had reached the cap stage of differentiation.

Because individual tooth germs had not been isolated as in the explants from 14-day embryos, not only tooth germs, but other structures also differentiated in the explants. In all cases, the oral epithelium differentiated as a keratinising stratified squamous epithelium as *in vivo*, but with a more rapid rate of development *in vitro*. On occasion, parakeratosis was noted, and epithelial pearls were a common finding in the explants and often distorted neighbouring structures. Meckel's cartilage and osteoid tissue developed, as also did the ducts of the submandibular or sublingual salivary glands. Such ducts contained material in the lumina which stained by the periodic-acid Schiff method.

c) Development of explants from 12-day embryos.

At 12 days in utero, the incisor and molar tooth germ primordia are represented by the individual dental laminae in the mandible (Fig. 13).

Fifteen explants were made. Some of them consisted of distal or proximal fragments of the mandible, and some were of the oral epithelium of the whole arch and its underlying mesenchyme.

Culture of such explants for 2 or 4 days gave rise to epithelial invaginations and mesenchymal cell condensations similar to those that represent a tooth germ at 14-15 days *in vivo*. Such invaginations, however, are difficult to differentiate from infoldings of epithelium thought to be due to distortion of the explants under the abnormal conditions of growth. In these explants, Meckel's cartilage, the lip furrow band, and the tongue with well defined epithelial pattern of the papillae, were found.

With 6 days in culture, the stage of cusp formation had been reached, and tooth germs were more easily identifiable. Early cusp formation was seen in one such tooth germ that developed in a molar region explant (Fig. 68). The external and internal enamel epithelia consisted of cuboidal cells, and the central cells of the enamel organ/

organ, although not stellate, were separated by intercellular material. The mesenchymal cells were not yet enclosed by the enamel organ, but were condensed and orientated as in vivo.

Histochemically, glycogen was present in the external and internal enamel epithelia, and less obviously in the dental papilla. The papilla also stained positively for neutral and acid mucopolysaccharide.

Such a tooth germ is very similar to that found in a 15½-day embryo in vivo (Fig. 23), the rate of development in vitro being, therefore, about 50% that of normal.

After 15 days in vitro, a molar tooth germ developed in which cusps L_1B_1 : L_2B_2 could be identified (Figs. 50, 68). The external enamel epithelium was composed of cuboidal cells, and the internal enamel epithelium of columnar cells whose nuclei were centrally placed. Little stellate reticulum was present. No odontoblasts could be identified in the papilla.

Glycogen was present in all cell layers of the enamel organ except for the internal enamel epithelium, and was also present in the dental papilla. The stellate reticulum stained positively for neutral and acid mucopolysaccharide, as did the dental papilla which stained more intensely than the surrounding mesenchyme.

Such a tooth germ resembled that of a 16-day embryo (Fig. 26). The rate of development - despite the obvious health of the explant - was only about 27% that of the normal rate.

In these longer-term cultures, as in the short-term cultures described, other mandibular structures developed as well as tooth germs. Osteoid tissue developed, and was found to be calcifying in vitro. Meckel's cartilage was also present in many explants. The salivary gland ducts seen were lined by a double row of cuboidal cells and contained periodic-acid Schiff staining material.

d)/

d) Development of explants from 11-day embryos.

It is at 11 days in utero, that the mouse embryo shows the first morphological signs of tooth development with the appearance of the dental lamina(Fig. 12).

Thirty-two explants were made consisting of fragments of mandible or the oral epithelium of the whole arch and its underlying mesenchyme.

With culture for 2,4 and 6 days, very good differentiation at almost 100% of the normal rate gave rise to tooth germs resembling those of 13-15 days *in vivo*.

After 8 days in culture, one explant developed an incisor tooth germ equivalent to that of the 15 days *in vivo* stage(Fig.22). In the same explant, M_1 and M_2 developed to a later stage of differentiation with cusps L_1B_1:L_2B_2:L_3B_3 being present in M_1. In this tooth germ, the external enamel epithelial cells were cuboidal, the stellate reticulum cells were widely separated by intercellular material, the stratum intermedium was 2-4 cells thick and the internal enamel epithelium was composed of columnar cells with centrally placed nuclei. Odontoblasts or predentine could not be detected.

M_2 was in the late cap stage of development, a slight increase in the intercellular material being seen centrally in the enamel organ.

This stage of development of M_1 and M_2 is similar to that of the 17-day stage(Fig. 30)- the rate of development *in vitro* being, therefore, about 75% that of normal.

Other explants cultured for 8 days, developed tooth germs which had developed to the 14-15 day *in vivo* stage, i.e. 50%of the normal rate of growth. They also developed salivary gland ducts, cartilage and tongue epithelium with the characteristic pattern of papillae.

In numerous explants where, after 2-4 days in culture, the molar region was isolated from larger mandibular explants and cultured separately, no molar tooth germs were found to develop, but in each case, salivary gland duct could be identified. It was thought/

thought that distortion of the tissues in culture had sufficiently altered the anatomical relationships of the structures to make identification of the tooth germ uncertain, and made them vulnerable to trauma.

After 14 days in culture, 4 recognisable tooth germs were found to have developed (Fig. 51). The teeth developed to different stages. In two explants they developed at about 50% of their normal rate to the 17½ day in vivo stage (see Fig. 45 and Figs. 28, 29). In another explant, both incisor and molar tooth germs developed to the 15½-16 day in vivo stage (Figs. 22, 23, 25, 26), i.e. at about 36% of their normal rate. This explant was cultured by the hanging drop technique for two days before being transferred to a watch glass. The histological differentiation of the molar tooth germ was similar to that of a 16-day embryo (Fig. 26), but histochemical staining showed glycogen to be present in the stellate reticulum - a condition not normally seen in vivo until the 17th. day (Fig. 45).

Salivary gland duct was seen in the explants cultured for 14 days. As in vivo, the cuboidal celled lining of the duct changed abruptly to the stratified squamous type near its entrance to the oral cavity.

e) Development of explants from 10-day embryos.

At 10 days in vivo, no morphodifferentiation of odontogenic tissue is seen in the embryonic mandible (Figs. 9, 10).

Nine explants were made, five consisting of whole mandibles from which the ventral epithelium had been removed, four also contained part of the rhombencephalon. Two of the explants were left undivided throughout the experiment, in another two, after 4 days, the incisors were isolated and subcultured separately. In one explant consisting of both arches, trauma in the Pasteur pipette resulted intheir separation at the mid-line, and each arch subsequently degenerated in culture. All other explants remained healthy during culture. The presence or absence of the rhombencephalon made little difference to the differentiation of the mandibular/

mandibular tissues *in vitro*.

On examination of the explants at each subculture, it was noted that after 4 days in culture, the epithelial thickenings for the incisor tooth germs and lip furrow band could be identified. As this stage is not normally seen *in vivo* until 12 days gestation age, a very approximate rate of development *in vitro* was assessed as at least 50% that of normal. Salivary gland epithelial invaginations were seen 6 days after setting up the cultures, and long rods of cartilage were apparent at 8 days *in vitro*.

Histological examination showed that after 9 days in culture, five of the explants showed the development of cartilage, salivary gland ducts, osteoid tissue, and keratinising stratified squamous epithelium which inone case showed parakeratosis. No tooth germs, however, could be identified with certainty in the folds of the epithelium.

In the other three explants, after 9 days in culture, 3 tooth germs were identified (Fig. 52). Two tooth germs appeared to have a molar rather than an incisor configuration. One of them was equivalent to the 15-day *in vivo* stage (Fig. 68), which gives a rate of development *in vitro* of about 55% that of normal. Numerous mitotic figures were present in the epithelium. The external and internal enamel epithelial cells were cuboidal, and the stellate reticulum showed increased intercellular spacing. Osteoid tissue was present near the cervical loop.

Histochemical staining showed glycogen to be present in the external enamel epithelium and stellate reticulum, and fewer granules in the internal enamel epithelium and dental papilla. Acid mucopolysaccharide was present only near the basement membrane in the papilla - but staining for neutral mucopolysaccharide in the papilla was not localised.

The other molar tooth germ was more advanced in development. The histochemical features were similar to those just described, but cusp formation was present, resembling an early 16-day embryonic molar (Fig. 26). The rate of development of this tooth germ in culture was therefore about 66% that of normal.

The/

The third tooth germ was elongated, and most resembled an incisor tooth germ at about the 15-day stage in vivo (Fig. 22), i.e. a developmental rate of approximately 55% that of normal (Fig. 53). No predentine was present, but the cells of the internal enamel epithelium were elongated at the incisal tip.

Histochemically, glycogen was present in the elongated cells of the internal enamel epithelium, but not at the cervical loop. It was present also in the papilla which stained positively for neutral and acid mucopolysaccharide.

f) Development of explants from 9-day embryos.

At 9 days in vivo, no morphological signs of tooth development are seen (Figs. 6, 7).

Eleven explants were made, each consisting of whole mandibles. Most explants were cultured without further dissection, but in a few cases, attempts were made to remove some of the ventral epithelium with finely drawn glass needles.

Of these explants, five developed well. Of those that failed to develop; one explant was deliberately made from the mandible of a noticeably retarded embryo in the litter, and it subsequently degenerated, and two mandibles were traumatised by the dissecting needle, and developed only into cysts in culture. Three mandibles were set up inculture after being stored in Tyrode's solution : horse serum for over 6-8 hours, while the litter mates were being used for another experiment. The three mandibles were roughly dissected, and cultured. A slight outgrowth of cells was seen after 2 days, but thereafter they quickly degenerated.

The explants were examined under the dissecting microscope at every subculture. It was noted that after 4 days, a tongue was apparent in each case, as well as epithelial thickenings for the incisor tooth germs and the lip furrow band. As stated earlier, the thickenings indicate a stage of development normally seen in the 12-day embryo - and here a developmental rate of about 75% that of normal was indicated. Long cartilaginous rods were obvious after 6 days in culture, and the presence of a possible tooth germ was/

was present after 10 days in culture (Fig. 64). In one of the explants which developed well in culture, a spherical tooth germ could be identified. However, the explant became infected before fixation and was useless for histological examination.

Four tooth germs developed, and were examined histologically. Two of these were in the same explant (Fig. 54). After 8 days in culture, one tooth germ was found to be narrower and more elongated than the other, but otherwise each had the same histological features of a cuboidal - celled external enamel epithelium, and an internal enamel epithelium where most of the cells were cuboidal, but those at the height of the bell tended to be more columnar. The stellate reticulum showed cells widely separated by intercellular material, and the stratum intermedium was 2-3 cells thick. One of the tooth germs showed a normal stellate reticulum, where the cells appeared truly stellate; the other showed more vacuolated cells in this region - a finding quite common in these cultures, and usually signifying impending degeneration.

Histochemical staining showed that glycogen was present in all layers of the enamel organ, except for the cervical loop region of both internal and external enamel epithelia of the more incisiform tooth. Glycogen granules were scattered less densely in the papilla than in the enamel organ. Fine collagen fibrils were present in the mesenchyme of the explants, but very few were present in the papilla. The dental papilla stained positively for neutral mucopolysaccharide, and the stellate reticulum stained positively with Alcian blue for acid mucopolysaccharide. No neutral mucopolysaccharide could be detected in the stellate reticulum.

These tooth germs, judging from the presence of columnar cells in the internal enamel epithelium at the height of the bell, and the histological state of the stellate reticulum, appear to be at the late 15 - early 16 - day stage (Figs. 22 - 27). The presence of Alcian blue staining in the stellate reticulum is indicative of a stage of development resembling that of 16 days in vivo. The approximate rate of development could be assessed at about/

about 75% that of normal. However, at the 16 day stage *in vivo*, cusps L_1B_1 : L_2B_2 are normally present in the embryo, and the cervical loop of the bell tends to enclose more of the underlying mesenchyme than is noticeable in the cultures. Some discrepancies between morphological and histological development seem apparent here.

After 10 days in culture, two tooth germs were found to have developed (Figs. 55, 56, 68). Their histological features were very similar to those already described after 8 days culture. The morphological features resembled a molariform rather than an incisiform tooth germ. Osteoid tissue had developed basally to the tooth germ as with the other cultured molars, rather than in the slightly lateral position it normally assumes with the cultured incisors of the older explants. The proximity of salivary gland ducts also indicated that molar tooth germs were present.

Histochemically, glycogen was present in all layers of enamel organ, except for the stellate reticulum, which tended to show signs of degeneration, as pyknotic nuclei. Glycogen, neutral and acid mucopolysaccharides were densely staining in the papilla. These tooth germs appeared to be at the 16-17 day stage (Figs. 25 - 30) - a 60% normal rate of development. However, once more, no cusp formation was present.

One explant showed no identifiable tooth germs, but as with the other explants, it showed salivary gland duct with periodic - acid Schiff staining material in the lumen, cartilage, calcifying osteoid tissue and stratified squamous epithelium, inwhich were seen epithelial pearls. The epithelium of the tongue showed the pattern of the papillae. The salivary gland duct opened on to the surface of the explant, being lined in this region by stratified squamous epithelium.

g)/

g) Development of explants from 8-day embryos.

This is the earliest age at which the mandibular arches are seen in the embryo. Both epithelium and mesenchyme consist of undifferentiated cells (Fig. 5).

Sixteen explants were made from this age of embryo and were of 4 different types:-

a) 6 were of the mandibular arches alone,
b) 1 was of the mandibular and hyoid arches,
c) 7 were of the mandibular arches and rhombencephalon,
d) 2 were of the mandibular and hyoid arches, and rhombencephalon.

Great care was taken in dissecting the explants. One of them, however, was damaged with a dissecting needle, and throughout 12 days in culture, it degenerated to form only a cyst (Fig. 62). All other explants, without exception, developed healthy tissues.

All the explants were cultured for at least 14 days, to allow any tooth germ that might have developed at approximately 50% of the normal in vivo rate, to be identified unequivocally.

From these 16 explants, only one tooth germ developed (Figs. 58, 68). It arose in one of the largest explants, and consisted of rhombencephalon, as well as the mandibular and hyoid arches. The explant developed well, showing long rods of cartilage and a tongue at about 6 days after explantation(i.e. an approximate developmental rate of these structures of 83% normal), and it was noted that a structure resembling a tooth germ was present at 12 days in culture. Fixation was at 19 days. Tooth germs were normally discernible in culture only when the enamel organ has enclosed the mesenchyme in a bell-shape, and the tooth germ resembles a sphere in which the closely packed cells of the internal enamel epithelium are refractile and identifiable. Such a stage is equivalent to that at 16 days in vivo, and therefore a very approximate assessment of rate of development in this case during 12 days in culture is 66% that of normal.

Histological/

Histological examination of the tooth germ, however, showed it to be neither a normal incisor nor a molar tooth germ. It was egg-shaped, bearing a slight resemblance to the overall shape of a 14-15 day incisor tooth germ *in vivo*, but its greatest volume was occupied by the enamel organ, not by the papilla as in the normal tooth germ. The enamel organ was almost entirely composed of a histologically normal stellate reticulum, except that it occupied an even greater proportion of the tooth germ than it does at its maximum volume in the normal molar tooth germ. The internal and external enamel epithelia could be seen to be composed of cuboidal cells. No cusp formation was present.

Histochemically, dense glycogen granules were present in all layers of the enamel organ, being especially dense in the external enamel epithelium. The dental papilla contained much sparser and finer granules. The papilla stained positively for neutral mucopolysaccharides, but none could be detected in the stellate reticulum.

The general development of all the explants was good (Fig. 57 and 59 - 61).

On all cultures, after 2 days it was noted that no thickening of the explants had taken place as was usual with the older explants, but that the fibroblastic outgrowth seemed to exceed the volume of the original explant. The excessive outgrowth was particularly apparent in the smallest, which contained only the mandibles, and in others which were slightly traumatised, e.g. by suction in the Pasteur pipette during transferrence to the cover slip. After the second day, the explants thickened, and the outgrowth was not so excessive.

Examination of the cultures, under the dissecting microscope each day, revealed cartilage for the first time at 4 days, and by 6 days it had grown to recognisable rods of tissue. In whole mandibles (i.e. 2 arches), the rods were seen to approach each other, in normal anatomical relationship, but even after 12 days in culture, they had not united to form the rostral process. The cultures usually rounded up after about 6 days, and the normal anatomical/

anatomical arrangement seen before this time was distorted - possibly accounting for a mis-alignment of the rods.

A tongue often became apparent, also at about 6 days in culture, and when it was present, was always placed centrally between the rods of cartilage as in vivo. In one explant, formed by the fusion of 2 whole mandibles, only one tongue, instead of the expected two, developed centrally - but this was not detectable by histological examination later.

It was noted repeatedly, that structure was most clearly visible within the explants at about 12 days in culture. Thereafter, the explants became more opaque, possibly due to keratinisation of the stratified squamous epithelium.

It was obvious from an examination of the living cultures, and from their photographs, that although the explants were deliberately cultured for 14-20 days to allow maximum tooth development to take place, in fact all tissues looked less healthy after about 16 days in culture. After this time they darkened centrally, rounded up more noticeably and showed little outgrowth of cells, despite roughening of the edges at each subculture.

Close attention was paid to development of structure in each of the four types of culture.

Histological examination of the development in culture showed that in all types of explant, cartilage, osteoid tissue, salivary gland and keratinising stratified squamous epithelium often showing epithelial pearls, were capable of development (Fig. 57 & 65). Inthe larger explants, fibro-cartilage as well as hyaline cartilage, was occasionally seen and also pseudo-stratified ciliated columnar respiratory epithelium, presumably of tracheal origin (Fig. 63).

Inthe two explants in which the hyoid arch was included, thyroid gland acini developed (Fig. 57, 66). Colloid was present, staining intensely with Eosin, and with the periodic-acid Schiff reaction, but not with Alcian blue. Resorption vesicles were present peripherally in the colloid in many of the acini. The explants which contained the glandular tissue were particularly healthy/

healthy, with few degenerate cells. Comparison with thyroid gland development *in vivo*, indicated that the closest resemblance was to the stage found normally about 2 days after birth.

In one of the explants containing the hyoid arch, groups of cells could be identified apparently in lobules divided by sparse connective tissue (Fig. 67). In one of the lobules, the cells were grouped about an irregularly shaped acinus containing material staining intensely by the periodic-acid Schiff reaction. Many of the cells were very large, almost double the average cell size, and had very clear cytoplasm. They most resembled the Wasserhelle cells of the young parathyroid gland.

DISCUSSION

From examination of the tissues which developed in cultured explants from successively younger embryos, it is clear that mandibles from the 10-14th days gestation age of the EZL strain of mouse are capable of self-differentiation in culture into recognisable tooth germs with the cusp pattern of the molar, or the elongated morphology of the incisor. Mandibular tissues explanted from 9-day old embryos are capable of forming tooth tissue, but cusp formation appears to be absent. Mandibular tissues explanted from 8-day old embryos, on the whole, seem incapable of tooth germ formation.

Features of the Development of Explants from embryos of 11-14 days Gestation Age.

All reports in the literature have shown that whether natural media were used (Hay, 1953b, 1956, 1961; Fisher, 1957; Glasstone, 1953, 1961; Pourtois, 1964; Kollar & Baird, 1968), or an artificial chemically-defined medium (Glasstone, 1965), mandibular tissues from all strains of mouse examined at 11-14 days gestation age, are self-differentiating into molar or incisor tooth germs in culture. The present results indicate that mandibular tissues from the EZL strain of mouse have the same capabilities (see Table III).

The reports have also shown that in many respects, the development of the tooth germs *in vitro* does not mimic exactly the development *in vivo*. A closer examination was therefore made of the rate of development, and the normality of the morphological histological and histochemical development *in vitro* of the tooth germs of the EZL strain of mouse.

Rate/

Rate of development. The most noticeable feature of the rate of development of the tooth germs in culture, was that tissues explanted from the same litter, or even the same embryo, cultured under almost identical conditions in the same experiment, did not develop at the same rate. This is a finding not confined to organ culture conditions alone. Slavkin & Bavetta (1968b) made the same observation on the rate of growth of rat tooth germs on the chicken chorio-allantoic membrane.

Small differences in the culture conditions may be responsible for differences in developmental rates, but a more likely cause would appear to be trauma : the smallest fragments of tissues, isolated from 11- and 12-day old embryos, subjected to most trauma of dissection developed at a noticeably lower rate than larger fragments or small whole mandibular arches; in one explant from a 14-day old embryo containing two incisor tooth germs, one of them adjacent to a region dissected off at the first subculture and therefore traumatised by the cataract knife, was retarded in development compared to the other one.

Differences in the histological development of *in vitro* and *in vivo* tooth development, make only a rough estimate of the difference in rates of development between the two, possible. However, taking the average development of morphological and histological appearances, a table of conservative estimates of rate of development in culture was compiled (Table II). On examination, it can be seen that the age of the embryo from which the explant was made, does not appear to affect the rate of development in culture. Hay (1956) made the same observation. Fisher (1957) and Pourtois (1964), however, formed different opinions. Fisher found that there was a decrease in the rate of morphological development varying with advancing age of the explanted tooth germ; Pourtois that there was an increase. Fisher's results may be explained by the fact that the oldest stages of development explanted by her, were very close to the maximum stage of development capable of being achieved in culture.

A/

A positive correlation was, however, found between the rate of development in culture and the duration of the culture period. The rate was found to be inversely proportional to the time in culture. No disagreement is found in the literature on this point (Hay, 1956; Fisher, 1957). Lefkowitz & Swayne (1956a), attempted to explain it by culturing the explants in embryo extract free medium, and concluded that there is'a residual nutrient medium in the organ, which until consumed is capable of sustaining growth and development'.

A study of "absolute" values of rate of development, as reported in the literature, is of interest. In the EZL strain, culture of tissues for short periods of time of 2-6 days, always gave rise to a rate of development of over 50% that of normal. Culture for 8-10 days gave a rate of 50-75% that of normal, and culture for 12-14 days varied from 27-50% that of normal.

By comparison, Fisher found that the rate of histo-differentiation in vitro was a little faster than the rate of morphological differentiation. She found that with explants from 13-14 day old embryos, the rate of histodifferentiation at the beginning was 100%, dropping to 60% in 10 days, and 57% in 14 days of culture. Morphodifferentiation rate after 10 days in culture fell to 50% that of normal.

Hay (1956) did not differentiate between the rates of morpho- and histo-differentiation. Comparing rates after 14 days in culture, the rate of development of explants from 10-day old embryos was 36%, from 11-day old embryos was 50-57% and from 12-day old embryos was 47-53%.

Pourtois (1964) did not specify the length of time in culture in comparing rates, but found that tissues from 9½ day old embryos developed at 40% of the normal rate, from 10½ day old embryos at 60-66% and from 12 day old embryos at 70%.

It would appear that under no conditions of culture so far achieved, does the rate of development of tooth germs approach closely that found in vivo.

Morphological/

Morphological development. There was no difficulty in identifying the major cusps L_1B_1 : L_2B_2: L_3B_3 : 4 in the cultured explants from the EZL strain of mouse. Extra foldings of the basement membrane had certainly developed, particularly on the labial wall of the incisor, and on the cusps of the molars, but they in no way caused confusion in identifying the molar and incisor morphology. Hay (1956) considered the crown pattern produced in vitro to be abnormal. Fisher (1957) made a close study of crown form, and concluded that the proportion of typical molar pattern which developed increased with the age of the explants: 70% in explants from 13-14 day old embryos and 86% from 18-19 day old embryos. The abnormalities she found resulted from fusion of cusps B_1 and B_2, and of cusp 4 with L_3 B_3. Glasstone (1938a, 1939) found that with cultured rabbit embryonic tooth germs, a small extra cusp was consistently present.

However, it has been pointed out by Kraus (1952) that minor cusps seen during development may be unidentified in the final morphology of the tooth because they are obliterated in the full width of enamel, and of even more significance, it was found that changes in the diet of the mother during pregnancy and lactation were sufficient to produce variations in the sub-division of major cusps, fusion of major and minor cusps, absence of minor cusps and bifurcation of minor cusps (Cox, Nathans & Wirth, 1952). Little value, therefore, seemed to be attached to detailing minor cusp abnormalities as a means of assessing either the adequacy of culture conditions or of cell potentialities in the present experiments. Only the identification of the major cusps was considered of significance.

Histological development. Examination of cultured tooth germs up to the time of deposition of hard tissue matrix showed that histodifferentiation seemed to follow the same sequence of development as in vivo, and that histological and morphological development were synchronised as in vivo. Fisher (1957) found that histodifferentiation appeared to develop at a faster rate than morphodifferentiation, but no evidence of this was seen in the present experiments.

At/

At the time of hard tissue matrix deposition, however, abnormalities of histodifferentiation were very obvious. Tubular predentine, and the osteodentine of the incisal tip, were laid down apparently as in vivo, but although deposition achieved considerable thickness, no calcification took place. Attempts to produce calcification by Hay (1961), by the addition of 0.2% calcium glycerophosphate to the culture medium produced only a thin shell of calcified material around the outside of the explant, whose innermost cells underwent degeneration.

No enamel matrix was observed in these cultures, even in tooth germs with a very advanced morphodifferentiation. On a closer examination of the reports of enamel production, by different authors, it was found that the 10% of mouse molars reported by Glasstone (1964) to form enamel, were at the relatively differentiated stage of 17 days at the time of explantation. The molar tooth germs that formed enamel (23% of the explants of M_1 and M_2), reported by Hay (1961), were explanted at the 15-18 day stage. No enamel is reported as developing in younger explants. As for incisor development, Hay (1961) found that enamel developed in 41% of explants from 12-18 day old embryos, but did not record whether the younger ages produced as many examples as the older ages. Koch (1965, 1967) found enamel almost invariably in his incisor explants from 16-day old mouse embryos, but (personal communication) he found none in any explant isolated before this stage.

As all the explants from the EZL strain were made before the 14-day stage, the absence of enamel formation during culture was to be expected in view of the preceding discussion, although some might have been expected in the older incisors. It may be significant that in the incisor tooth germ cultured from a 13-day old embryo mouse (Fig. 49) and treated by the modified Elftmann technique to stain the Golgi apparatus, the apparatus in all cells of the internal enamel epithelium was in a paranuclear position.

The/

The light microscopic studies of Beams & King (1933) and Ramadam Sadek (1962), and more recently, the electron microscopic studies of Frank & Nalbandian (1967) show that just before enamel matrix formation in vivo, the Golgi apparatus in the pre-ameloblasts moves from the paranuclear position to the functional (basal) pole of the cell. This is said to take place after calcification of the dentine matrix has begun, and whilst enamel precursors are seen to aggregate within the pre-ameloblasts. Perhaps it is not surprising that, as neither the calcification of the dentine nor the deposition of the enamel has been observed in the experiments under consideration, the internal organization of the cell is also abnormal.

It cannot be assessed from the experiments whether the abnormalities are due to lack of determination in the explanted tissues, or due to inadequacies of the culture medium to allow the potential to be expressed. The reports in the literature, however, are strongly suggestive that tissues explanted before 16 days of age for the molar, and 12 days of age for the incisor, are not determined for enamel matrix formation and calcification of dentine.

Thick layers of prismatic enamel have been reported as a common finding in transplanted tooth germs (Huggins, McCarroll & Dahlberg, 1934; Willis, 1935; Fleming, 1952) and in tooth germs grown on the chicken chorio-allantoic membrane (Glasstone, 1953; 1954b; Slavkin & Bavetta, 1968b). All authors attribute the superiority of quality and quantity of enamel found under these conditions, to the presence of the vascular supply not present in organ cultures. However, although possibly true, it does not prove that the transplantation conditions allowed the cells to demonstrate a state of determination for enamel formation which the culture conditions suppressed. All the tooth germs transplanted in the experiments reported were found to be in an advanced state of differentiation at the time of isolation, and would have been expected to show some enamel formation under organ culture conditions as well.

That/

That the cells are able to modulate and yet retain their ability to form enamel, was found by Main (1966). The incisors of 14-day old mouse embryos survived under culture conditions in gelatin sponges for 37 days, showing no morpho- or histo-differentiation as tooth germs. They were thereafter transplanted to isologous hosts, and within 56 days a thick layer of normal pigmented enamel had been formed by the cells. Presumably the cells isolated from the 14-day old embryos were already determined for enamel formation, but the culture conditions had not allowed the expression of this state to be demonstrated.

Histochemical development. Little work has been reported in the literature on the development of cytochemical features of tooth germs grown *in vitro*. Pourtois (1966) studied the development of the intracellular enzymes, alkaline and acid phosphatase, succinic dehydrogenase and also ribonucleic acid and collagen fibril formation in cultured tooth germs, but did not study the pattern of distribution in different parts of the tooth. Glasstone (1965) explanted tooth germs at a stage of development when no alkaline phosphatase was present, and found that despite the lack of calcification of the dentine, the developmental pattern for this enzyme *in vitro* was as *in vivo*.

In the present experiments, the development in culture of glycogen, acid and neutral mucopolysaccharides and collagen was studied. Due to the impossibility of staining every section with every stain, significant regions may not always have been represented, and care is necessary in interpreting the results.

Glycogen. In the cultured tooth germs, glycogen very often did not observe the precise localisations that were found *in vivo*. For example, the normal molar tooth germ showed glycogen mainly in the buccal wall of the external enamel epithelium at the 15-, 16- and 17-day stage; *in vitro*, the granules were not localised to this wall, but were also found lingually in the cell layer. At 19 days *in vivo*, glycogen disappeared from the stellate reticulum, but increased/

increased in concentration in the stratum intermedium. In vitro, glycogen appeared to be scattered indiscriminately throughout these tissues.

Both in vivo and in vitro, however, at all stages the cervical loop region of the enamel organ remained free of glycogen granules, and at the 19-day stage, the concentration of glycogen elsewhere in the external enamel epithelium increased. Some discrepancies also existed between different tooth germs in culture. In most cases, glycogen granules were a feature of the elongating cells of the internal enamel epithelium. In two cases, although the cells appeared elongated at the cuspal tips, no glycogen was present. It is possible, but unverifiable in the present explants, that the cells lacking glycogen were destined to overlie enamel-free areas and therefore lacked some of the developmental features of the future enamel-forming cells.

Localisation of glycogen in the dental papilla was also abnormal. In tooth germs at the 15-16 day stage in vivo, glycogen granules were not found in the developing dental papilla, but were found as a halo surrounding the tooth germ in the cells of the future dental follicle. At the equivalent in vitro stages, the granules were not confined to the periphery of the papilla, but were scattered throughout it.

In vivo and in vitro, tooth germs were alike in that the granules scattered throughout the dental papilla were finer and sparser than they were in the enamel organ, or in the surrounding connective tissue, and that in the well-developed tooth germs, the granules were denser in the cuspal regions or near the incisal tip of the papillae than near the cervical loop.

Neutral mucopolysaccharide. Whereas any glycogen that was found in the cultured tooth germs had developed de novo, neutral mucopolysaccharide was already present in all explants isolated after the 11th day in utero. The distribution/

distribution of the mucopolysaccharide in the cultured tooth germs was at no time different from that in the equivalent *in vivo* stage. It was occasionally difficult to identify in the cultured enamel organ, probably because it is normally at its densest at the time of matrix formation - a stage not reached in many of the cultures. It stained more intensely in the dental papilla than in the enamel organ in all tooth germs that developed in culture to the equivalent of the 16-day stage *in vivo* and beyond. Predentine stained positively for neutral mucopolysaccharide as *in vivo*.

Acid mucopolysaccharide. All tissues explanted from 11-14 day old embryos, already contained intercellular acid mucopolysaccharide near the basement membrane. During development, its distribution spread, but at no time differed in the cultured tooth germs from the equivalent stages *in vivo*, The dental papilla stained more intensely than the surrounding connective tissue in all explants equivalent to and older than the 16-day stage *in vivo*. Its presence in the stellate reticulum was more easily detected than the neutral mucopolysaccharide in that position. Predentine stained positively for acid mucopolysaccharides as *in vivo*.

Collagen fibril distribution. In general, the distribution of young collagen fibrils was very similar in cultured and normal tooth germs. Cultured tooth germs showing cusp formation, stained for collagen, showed a mass of very fine young fibrils in the dental papilla, dense in the cuspal regions, and particularly dense under the developing odontoblasts. Some discrepancies were noted between the *in vivo* and *in vitro* findings, in incisor tooth germs. *In vivo*, collagen fibrils increased in density and calibre throughout the dental papilla with increasing age. In the cultured tooth germs, in all cases, as *in vivo* the calibre and/

and density of the fibres were greatest at the incisal tip and around developing odontoblasts. In some tooth germs, however, collagen fibrils were seen throughout the papilla and in the cervical loop region as *in vivo*, but in others, despite staining in the connective tissues elsewhere and at the incisal tip, there was a complete absence of staining at the cervical loop and middle third regions. Predentine as *in vivo* stained vividly for the presence of collagen in the matrix.

Features of the Development of Explants from embryos of 8-10 days Gestation Age.

The preceding discussion was concerned with the development of explants isolated from embryos of 11-14 days gestation age. As we have seen, numerous reports have confirmed the self-differentiation in culture of mandibular odontogenic tissues from these ages. This, however, is not the case with explants isolated from 8-10 day old embryos - Hay (1956) and Pourtois (1964) publishing the only available reports.

In general, development of explants in culture from embryos of 8-10 days gestation age of the EZL strain of mouse, was very good. In all cases where degeneration took place, trauma or storage in adverse conditions appeared to be contributory causes. The theories of Losee (1943), Szabo (1954) and Pourtois (1964) that trauma was the principal cause of lack of development in cultures from young embryos, seems to be substantiated.

Development of explants from 10-day embryos.

The results of experiments where odontogenic tissues were explanted from 10-day old embryos of the EZL strain of mouse, showed that three tooth germs developed in the eight cultures made. The rate of growth of the tooth germs resembled those of cultures from older embryos, in that they differed for each explant, and covered a comparable range (55-66% that of normal). One molar and an incisor showed the typical morphology of normal tooth germs of the 15-day old embryo, and the other molar tooth germ showed early cusp formation/

formation characteristic of the tooth germ of a 16-day old embryo.

Histochemical staining of the tooth germ whose morphology resembled the molar of the 15-day old embryo, showed glycogen to be present in the external and internal enamel epithelia, and also in the stellate reticulum. Glycogen is not normally seen in the stellate reticulum *in vivo* until the 16-day stage, therefore either the cytochemical differentiation had proceeded more rapidly than the morphodifferentiation, or cusp formation was retarded. The dental papilla also stained for glycogen - seen in the other cultured tooth germs in this position at this stage, but not normally present within the dental papilla until the 17-day stage.

Neutral mucopolysaccharides were present in the dental papilla as *in vivo*, and acid mucopolysaccharides were found localised near the basement membrane - seen usually in this position earlier in development *in vivo*.

The more fully differentiated molar tooth germ showed a very similar histochemical picture to that just described, except that the acid mucopolysaccharides in the dental papilla were not localised at the basement membrane.

The incisor tooth germ showed glycogen in all cell layers of the enamel organ, and in the dental papilla as *in vivo*. Acid and neutral mucopolysaccharides were present in the dental papilla, also as usual.

It would appear that the three tooth germs which developed in the present experiments had reached a comparable stage to the molar developed from a similar age in the experiments of Hay (1956). She described it as showing early cusp formation and differentiation of the cells of the internal enamel epithelium. No recognisable tooth germ is available for comparison in the experiments with this age reported by Pourtois (1964).

The results of these experiments with the EZL strain, and from Hay's report, suggest that the mandibles from 10-day old embryos are capable of self-differentiation in culture into tooth germs with the early cusp formation of the molar, or the elongated shape of the incisor.

Development/

Development of explants from 9-day embryos.

When odontogenic tissues were explanted from embryos of 9 days gestation age, four tooth germs developed in five healthy explants. There seems little doubt, therefore, that mandibles from 9-day old mouse embryos of the EZL strain are capable of self-differentiation in culture into tooth tissue. On closer examination, however, it could be seen that the morphology was abnormal. In all cases the cervical loop region was unusually wide and no cusps were present. The tooth germs that developed after 8 days in culture gave the histological and histochemical picture of a tooth germ equivalent to the 16-day *in vivo* stage, when cusps L_1B_1: L_2B_2 could have been expected. The external and internal enamel epithelia were composed of cuboidal cells, and the stellate reticulum contained much intercellular matrix. All cell layers of the enamel organ contained glycogen except at the cervical loop region- and the stellate reticulum stained for acid mucopolysaccharide. However, no neutral mucopolysaccharide could be detected in the stellate reticulum despite a strong staining reaction for it in the dental papilla. The dental papilla contained fine collagen fibrils, and stained positively for glycogen and acid and neutral mucopoly-saccharide as *in vivo*.

The tooth germs that were seen after 10 days of culture had similar morphological and histological features, except that the stellate reticulum did not look so healthy in these cases, and although neutral mucopolysaccharide was present, there was no glycogen.

To ensure that the capacity for cusp formation is absent, and not merely retarded, longer periods of culture would seem to be advisable.

Nevertheless, the results indicate that at the time of isolation, only the first part of the pattern of morphological development was determined in the odontogenic cells. It has been established by Ramadan Sadek (1962) that dividing cells exist in the greatest numbers at the cervical loop region of the internal enamel epithelium/

epithelium at the bud and early cap stage of development, and it is by growth at the cervical loop region that the walls of the tooth germ develop and the bell-stage achieved. His painstaking work has verified the less detailed observations of Santoné (1935), Lefkowitz, Bodecker & Mardfin(1953), Baume, Becks & Evans(1954) and Paynter & Hunt(1961).

Examination of the tooth germs that developed in the explants isolated from the 9-day old embryos suggests that the pattern of increased mitotic activity in the cervical loop region does exist.

It is at the late cap stage of development that the future cuspal regions of the molar tooth germs are mapped out. Ramadan Sadek(1962) verified the similar observations of Blechsmidt(1953) and Butler(1956) and identified at this stage bands of mitoses that form the main transverse valleys(sulci) of the crown. These bands of mitotic figures did not appear in the cultured tooth germs under consideration. It would appear that at the time of isolation, only the pattern for development up to the late cap stage was determined in the cells, and the determination for the fine details of cusp formation was, as yet, absent. The determination was acquired, as we have seen, during the following 24 hours: odontogenic tissues isolated from 10-day old embryos and cultured, formed cusps.

Although determination of the cells for cusp formation is not expressed as active histodifferentiation until about 5 days later, at the late cap stage of development, Fisher(1957) by her experiments with halved tooth germs, has shown that the state of determination remains plastic almost throughout the latent period, until the early cap stage(14 days gestation age).

Development of explants from 8-day embryos.

When odontogenic tissues were isolated from 8-day old embryos and cultured, only one of fifteen healthy explants developed a tooth germ. After 19 days in culture, no cusp formation was detectable and in this, as in the histological and histochemical appearances, the tooth germ resembled those developing in explants from/

from the 9-day old embryos : the cells of the internal and external enamel epithelia were cuboidal, and the stellate reticulum was well differentiated. The dental papilla was very small. Histochemically, glycogen was present in all cell layers, though much sparser in the stellate reticulum than elsewhere in the enamel organ. As with one of the explants from the 9-day stage, no neutral mucopolysaccharide was detectable in the stellate reticulum.

Allen & MacDowell (1940) have recorded the well known fact that within any litter at the 8-9 day stage, some mouse embryos are noticeably more advanced in development than others. As fourteen of the fifteen explants developed healthy tissues in culture, but no tooth germs, it is suggested that 8-day old embryonic mouse mandibles are not determined for tooth formation, but that the ability is rapidly acquired and the one embryo from which a tooth germ was cultured, was more fully differentiated at the time of explantation than the others.

However tempting it is to assume that failure of tooth germs to develop in culture stems from lack of determination in the cells at the time of explantation, there still remains the possibility that the artificiality of the culture conditions interfered with the expression of any determination that was present.

It has been shown repeatedly (reviewed by Grobstein, 1965), that for complex histogenesis to take place in culture, the mass of the isolated primordium must exceed a given level. The mass of the mandible of an 8-day old mouse embryo (in itself only about 1.5 m.m. CR length) is small, and is decreased even more by the rapid emigration of cells from it early in culture. The cell migration is possibly of dual origin:- 1) cells are known to emerge more rapidly on explantation from young embryos than from older embryos (Moscona, Trowell & Willmer, 1965), and 2) lack of contact inhibition, itself brought about by the smallness of the explants, further encourages the rapid migration of the peripheral cells (see Abercrombie, 1965).

In/

In support of the adequacy of the culture conditions for the expression of determination for tooth formation, is the fact that not merely some,but all other structures normally found in the mandible were found to be capable of differentiation - epithelia of different types, cartilage, osteoid tissue, salivary and thyroid gland with parathyroid. The normality of development of these structures under the present conditions was studied, and will be described in turn.

a) Differentiation of epithelium. All oral epithelium, no matter at what stage of embryogenesis it was explanted, differentiated as *in vivo* to keratinising stratified squamous epithelium, although, as has been found in other situations, it developed more rapidly than *in vivo* (Miszurski, 1937; Porter,1960). Parakeratosis was found on occasion, and epithelial pearls were a very common finding, especially in the explants from the younger embryos of 8-10 days gestation age.

Explants isolated from 9-day and older embryos, showed histologically the papillary pattern of the surface mesenchyme and epithelium of the tongue. The pattern could not be identified in the explants cultured after isolation from 8-day old embryos, suggesting that the tissues acquire their determination for such a pattern during the 9th day of age.

In the larger explants from the 8-day old embryos, in which the pharynx was included, respiratory type epithelium- ciliated pseudo-stratified columnar epithelium- differentiated. Porter (1960) found that only when the explants were isolated from older (19-day) rat embryos, did goblet cells differentiate in respiratory type epithelium, but this was not the case in the present experiments; nor did the excess Vitamin A in the fowl plasma seem to suppress the presence of goblet cells in the cultures, as found by Aydelotte (1965): they seemed as numerous *in vitro* as *in vivo*.

b)/

b) Differentiation of Meckel's Cartilage. The cartilage that developed in all cultures from the embryos of 8-10 days gestation age, and in the fragments of the mandibles from older embryos, was of the typical hyaline type as found *in vivo*. The histological appearances suggested that chondrogenesis proceeded as outlined by Fell (1925), i.e. by division of the chondroblasts, followed by secretion of the intercellular material and hypertrophy of the ageing cells. *In vivo* and *in vitro*, the dividing cells and those secreting matrix, stained intensely for glycogen, whilst the ageing hypertrophic cells contained very little.

In some of the long term cultures, endochondral ossification was seen. The first signs of endochondral ossification of Meckel's cartilage at the mental foramen region, are normally seen in the EZL embryo at 16 days gestation age, and in most cultures it seemed that the process was slightly delayed.

The results show that from the time of the first appearance of the mandibular arches, the tissues are self-differentiating into cartilage on culture. Cartilaginous rods appeared in culture of the mandibles from 8-day old embryos whether the arches were explanted alone, or were part of a larger explant which included the rhombencephalon. In the EZL strain of mouse, no histological or histochemical signs of the anlage of Meckel's cartilage are seen at this time - the first signs appear one day later, when cells following the former track of the first arch of the aorta stain positively for glycogen, and throughout the following days, differentiate into cartilage. Milaire (1959) and Pourtois (1961), in their strains of mouse, also identify the anlage of Meckel's cartilage in this position by the presence of alkaline phosphatase and glycogen in the cells. However, they also identify these cells as prolongations of the neural crest cells (ectomesenchyme) from the Gasserian ganglion, and thus attribute a neural crest origin to Meckel's cartilage.

The/

The fact that the tissues are self-differentiating into Meckel's cartilage one day before the apparent influx of the ectomesenchyme into the mandible, must mean that a) Meckel's cartilage is not composed of neural crest cells, or b) that the appearance of glycogen does not signify the first identifiable signs of the neural crest - but that the cells have already entered the arch undetected, or c) that, as with the induction of chick somite cartilage, only an impulse of induction (in their case from the spinal cord and notochord) is necessary for the cells to become determined for cartilage formation (Avery, Shaw & Holtzer, 1956; Lash, Holtzer & Holtzer, 1957; Holtfreter, 1968; Holtzer, 1968).

The formation of cartilage in culture from this age of embryo, is significant, in that according to Milaire (1959) and Pourtois (1961) the cells of the neural crest are responsible for both Meckel's cartilage and tooth germ formation. In these explants from the EZL strain Meckel's cartilage formed but tooth germs did not.

c) Differentiation of osteoid tissue. Osteoid tissue was a common finding in all cultures. In practice, its presence was frequently used to identify rapidly the position of an early epithelial enamel organ, as it was found that the two were often closely associated. In the long term cultures, it frequently calcified, despite lack of calcification of predentine matrix. The finding of bone around tooth germs is not confined to organ culture, but to growth of tooth germs on the chick chorio-allantoic membrane (Hoffman, 1967), and in transplantation (e.g. Palazzi & Bugliari, 1940; Nuckolls, 1941; Hoffman, 1967) and it has come to be assumed that the developing tooth germ in some way either induces or models the alveolar bone around it. Experimental proof, is however, still lacking.

d)/

d) Differentiation of salivary gland tissue. The presence of salivary gland tissue was a common finding in the larger explants made of mandibular tissues from embryos of 8-14 days gestation age.

From all ages, the development of the glands in vitro was similar to that described by Borghese (1950a) of rudiments isolated and cultured from mouse embryos of 13-15 days of age : a maximum stage was reached in all long-term cultures where the acini were abundant and solid, and the intraglandular ducts were hollow; the ducts were of two types, one with an irregular epithelial lining containing occasional goblet cells (submandibular glands), the other with a regular epithelial lining. Both types of duct showed mucous secretion, despite the fact that in vivo, the submandibular gland is a purely serous gland. The main duct, as was also found by Borghese, showed extreme dilation on occasion.

Rudiments isolated before the stage of the initial impocketing of mouth epithelium, in the experiments of Grobstein (1953a), and before the stage of representation as a stalk and terminal bud in the experiments of Borghese (1950a) did not develop. In the present experiments, no isolated rudiments were explanted, the glands developing only as part of larger explants. The fact that they developed from tissues explanted as early as 8 days gestation age, 4 days before the first histological signs are seen, supports the theory of Borghese (1950b), that a certain minimum size of explant may be necessary for the normal development of the tissue.

In the EZL strain of mouse, the mandibular tissues appear to acquire their determination for salivary gland formation during the 8th. day of gestation.

e) Differentiation of thyroid gland tissue. It was noted that in all explants from 8-day old embryos, where both mandibular and hyoid arches were explanted together, thyroid gland differentiated. During the 14-19 days in culture, the tissues developed from a completely undifferentiated state as seen by the histological and histochemical methods employed, to a stage equivalent to the 2-5 day old mouse in vivo where similar colloid-containing acini are present.

Boyd/

Boyd (1964) and Shepard (1965) suggest that induction is mediated by the truncus arteriosus rather than by the mesenchymal cells, but experimental proof is lacking, and the present experiments do no more than indicate that the glandular tissues are determined at this very early stage of development.

Not only was colloid present in the acini, but the histological appearances suggested that the gland was active. The presence of colloid is not indicative of activity on the part of the gland (Hall & Kaan, 1942), but the presence of peripheral vacuolization within the colloid (Carpenter & Rondon - Tarchetti, 1957; Boyd, 1964; Shepard, Andersen & Andersen, 1964) and more especially the presence of colloid droplets in the acinar cells (Stein & Gross, 1964; Wissig, 1964) indicates active resorption in response to stimulation by thyrotrophic hormone. The peripheral vacuoles and intracellular droplets were a prominent feature of many of the cultured acini. Lasnitzki (1965) noted that thyrotrophic hormone was present in natural culture media, and Carpenter & Rondon-Tarchetti (1957) showed that embryonic rat thyroid is capable of binding iodine in culture. Furthermore, the results of experiments by Ebeling (1924) and Gaillard (1955) suggest that thyroid gland is capable of activity in culture. Ebeling found that fibroblasts explanted beside thyroid gland showed increased growth and migration, and Gaillard found that thyroid gland in culture stimulated osteoblastic activity in adjacent explants. Gerstner & Butcher (1958) reported that thyroid gland of unspecified age, explanted beside tooth germs in culture, improved the rate and extent of tooth germ maturation.

Thyroid gland within the explants from the 8-day mandibles in the present experiments, certainly formed part of very healthy cultures with well-differentiated epithelium, cartilage and osteoid tissue. Many of the explants without the glandular tissue, however, also showed good differentiation of structure. Certainly the presence of active thyroid gland played no part in inducing tooth germ formation.

f)/

f) Differentiation of parathyroid gland tissue. In one culture from an 8-day stage mandible, where the explant included mandibular and hyoid arches and rhombencephalon, a group of cells arranged in lobules divided by scant connective tissue was found. The cells appeared to resemble mainly the primordial and vesicular cells of the parathyroid gland (Norris, 1937, 1946) and in one lobule, were Wasserhelle cells surrounding a mass of colloid, staining intensely by the periodic-acid Schiff reaction. Cowdrey (1938) noted that such colloid is said to differ from true thyroid colloid in the absence of iodine. It would appear that the cephalic anterior face of the third branchial pouch was inadvertantly included in the explant. Gilmour's observation (1937) that the water clear cells are associated with parathyroid III, tends to support this theory. Although Gaillard (1955, 1961) has shown that parathyroid gland from 2-4 day old mice in culture has a direct action on skeletal tissue, causing bone resorption, no such evidence of activity was found in the present experiment.

The normality of development and the diversity of the structures developing in mandibles isolated from 8-day old embryos and cultured, strongly suggests that although the original explants were small in mass, complex histogenesis was possible. Had the explanted tissues been determined for tooth formation - as they were for salivary gland, thyroid gland and parathyroid gland - then it seems very likely that differentiation would have taken place under these conditions of culture.

A Consideration of Factors Thought to Influence Tooth Formation in the Mouse Mandible.

We must now turn to the precise way in which determination is brought about.

Inducing factors which bring about determination of tissues for specific ontogenesis are as yet unknown - nor do the *in vitro* cultures described above throw any light on such factors in tooth development. The influence on the chain of events leading to the initiation of tooth formation played by the blood supply, basement membrane/

membrane, mesenchymal cell condensation and neural crest cells, has been discussed in Part I. These features were thought by histologists to be significantly placed in position and in time to be involved in the chain of reactions in early tooth development. In the organ culture experiments described, some of the features were affected by the isolation of the explants from the rest of the embryo, and it is of interest to consider if the hypotheses of their involvement based on histological data are upheld or refuted by the experimental data.

a) The blood supply. Mandibles isolated from embryonic mice of the EZL strain at 8 days of age had no blood supply. At 9 days gestation age, the blood supply was represented by the first arch of the aorta, and at 10 days gestation age it had been replaced by a mass of capillaries.

The complexity of the blood supply to the mandible therefore increases as the complexity of developmental pattern of tooth determination. These results are not inconsistent with the hypothesis that the blood supply is directly influential in early tooth development, but nor do they actively support the theory. The simplest and most plausible explanation is that the enlarging and differentiating mandible benefits from the more efficient method of energy supply achieved by the establishment of a ramifying vascular system. Once explanted, the tissues are no longer dependent for their energy on a blood supply, but derive it from the nutrients supplied in the culture medium. The hypothesis already discussed (page 42) that where there is a limited supply of energy (distributed by the blood vessels in vivo) the tissues enter into competition for it, may well apply also under culture conditions as some of the experiments suggest. It was noted that where whole mandibles were cultured from 9, 10 and occasionally 11-day old embryos, at no time did the full complement of teeth develop. Instead of four or six crowded or diminutive tooth germs in each jaw, either one or two well-differentiated and reasonably sized tooth germs developed in each explant. It may be that the nutrients supplied were insufficient for development of all tooth germs/

germs normally found *in vivo*. An "all-or-none" phenomenon as to whether there is a well-formed tooth germ or not, may be assumed if the hypothesis of Grüneberg (1966) is tenable and that there is "a minimum size below which tooth germs would regress."

b) The basement membrane. The organ culture experiments throw no light on the role in development of the basement membrane, as it was not disturbed in odontogenic regions during explantation, and at no time during culture did it seem to alter from its state *in vivo*.

c) The mesenchymal condensation. The individual mesenchymal cell condensations for molar and incisor tooth germs appeared gradually *in vivo* at the 13-14 day stage. All explants isolated immediately before and at these stages developed similarly *in vitro*. To assess any direct effect on future development, the mesenchymal condensation would have to be isolated from the odontogenic epithelium and experimentally recombined before culture - a procedure discussed in Part III of the thesis.

d) The neural crest cells. In the EZL strain of mouse, the cells with strongly basophilic cytoplasm, identified by Dalq (1953), Milaire (1959) and Pourtois (1961) as of neural crest origin (ectomesenchyme) were seen in the oral half of the mandible at the 10-day stage. Meckel's cartilage, identified also by them as being of neural crest origin, was seen first as a condensation of cells rich in glycogen proximally in the mandibular arches at the 9-day stage. No evidence of any such cells was present at the 8-day stage. As with the blood supply, the increasing invasion of the mandible arch by ectomesenchyme, parallels increasing complexity of developmental pattern for tooth formation. Once more, however, there is no proof of interdependence between the two.

In an attempt to find such proof, the explants isolated from 8-day old mouse embryos were essentially of two types: those composed of the mandibular arches alone, from which it was hoped all ectomesenchyme was excluded; those composed of the mandibular/

mandibular arches together with the rhombencephalic region, from which it was hoped that neural crest cells would migrate during culture into the mandibular arches. The results indicated that in neither case did tooth germs develop.

Numerous explanations for such a result present themselves: those assuming that ectomesenchyme did enter the mandible, and those assuming that it did not.

The enigma that Meckel's cartilage did develop in explants from 8-day old embryos, whilst the tooth germs did not, has already been discussed (page 126). If the origin of the cartilage as ectomesenchyme were convincingly established, then it follows that trigeminal ectomesenchyme was present in all explants under consideration. The situation, however, is in no way clarified by the recent study of Kollar & Baird (1968) in which tooth germs failed to develop in a medium containing the specific ectomesenchyme inhibitor beta-2-thienylalanine (Wilde, 1955; Papaconstantinou, 1967). Cartilage was unaffected by the inhibitor - as was bone and skin development. Assuming for the present that the cartilage is of ectomesenchymal origin, then the lack of formation of tooth germs may be attributed to:

1) lack of competence of the odontogenic ectoderm to react to the inducing influence of the ectomesenchyme,

2) lack of correct positioning of the ectomesenchyme, due to the artificiality of the conditions,

3) as the ectomesenchyme for Meckel's cartilage differs from the ectomesenchyme for odontogenesis, by having a high glycogen rather than a high cytoplasmic ribonucleic acid content (Milaire, 1959; Pourtois, 1961), there is the possibility that the conditions may have favoured the development of one, but not the other.

On the other hand, odontogenic ectomesenchyme may not have been included in the explants at all. Bartelmez (1962) found that neural crest cells entered the maxillo-mandibular region from the forebrain/

forebrain - a region definitely not included in the explants under consideration. Adelmann (1925) suggested that in rat embryos the trigeminal neural crest loses its attachment to the mid-brain and retains it to the rhombencephalon at about the 14 somite stage. The stage at which I excluded the mid-brain from the explants of mouse tissue was between the 10-15 somite stage, therefore there is the remote possibility that essential ectomesenchyme was excluded with the mid-brain.

To summarise it would seem that no direct correlation can be traced between early tooth formation, and such factors as the mesenchymal cell condensation, the presence of the basement membrane, and the invasion of the mandibular arch by the vascular system and by ectomesenchymal cells.

SUMMARY

Mandibular tissues from mice of 8-14 days gestation age were explanted and cultured under organ culture conditions. Tissues from 10-14 day old embryos were self-differentiating in culture into tooth germs. The tissues were therefore assumed to be determined for such formation at the time of explantation. They differentiated in long term culture to a maximum stage of development where the typical molar or incisor morphology could be identified, where early histodifferentiation resembled that *in vivo*, but where calcification of dentine matrix and deposition of enamel matrix was missing. The significance of these abnormalities was discussed.

Histochemical development differed in some cases from that *in vivo*, as far as glycogen distribution was concerned, but no gross abnormalities were noted.

Tissues from 9-day, and late 8-day old embryos appeared to be determined only for the early part of tooth morphological pattern, in that the fine details of positioning of mitotic activity for cusp formation was not expressed in culture. Other morphological, histological and histochemical features were similar to those of the older explants.

Tissues from 8-day old embryos would seem to have no determination for tooth formation, but Meckel's cartilage, salivary gland, thyroid and parathyroid gland differentiated characteristically.

The roles of the blood supply, basement membrane, mesenchymal cell condensation and neural crest cells in induction, were re-assessed in the light of the experimental data. The increasing/

increasing complexity of determination for tooth germ formation was found to parallel the complexity of development of the blood supply to the mandible, and the degree of infiltration of ectomesenchyme, but no proof of interdependence of any of the factors was found.

TABLES II & III

&

FIGURES 47 - 68.

$$\text{Rate of Development} = \frac{a - b}{c} \times 100\%$$

Where a = Nearest equivalent stage *in vivo*, to stage of development achieved by cultured tooth germ (days).

b = Stage of development *in vivo* at time of explantation (days).

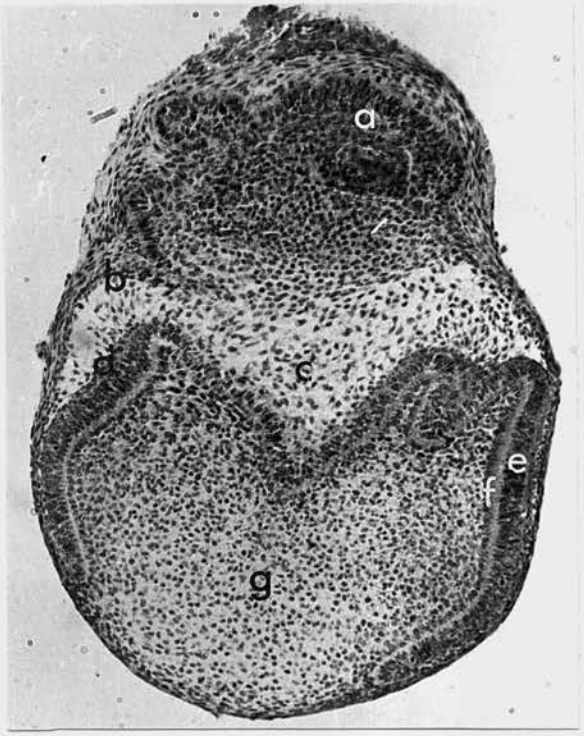
c = Number of days in culture.

TABLE II

Rates of Development of Tooth Germs *in vitro*, as Compared to Rate of Development *in vivo*.			
Age of embryo from which tissues explanted	Type of Tooth Germ	Duration of culture (days)	Rate of Development - as %age of Rate *in Vivo*
14 days	Incisor	6	42%
	Incisors	12	33%
	Molars	12	46%
13 days	-	2, 4, 6	50%
	Molar	8	50%
	Incisor	14	36%
	Molar	14	43%
12 days	-	2, 4	>50%
	Molar	6	50%
	Molar	15	27%
11 days	-	2, 4, 6	50-100%
	Molars	8	75%
	Incisor	8	50%
	M + Is.	14	50%
	M + Is.	14	36%
10 days	Molar	9	55%
	Molar	9	66%
	Incisor	9	55%
9 days	M + I	8	75%
	M + I	10	60%
8 days	M + I	19	-

TABLE III.

Numbers of tooth germs developing in explants which survived.						
Age of embryo from which tissue explanted	No. of explants cultured	No. degenerat^{g} due to trauma, adverse conditions	Reason for degenerat^{n} where known	Surviving healthy explants	No. of explants developing tooth germs	% of healthy explants in which teeth developed
14 days	6	-	-	6	6	100%
13 days	21	7	Dissection during subculture	14	10	71%
12 days	15	4	Dissection at explantat^{n} and subculture	11	8	73%
11 days	32	16	Bad dissection Bad conditions	16	12	57%
10 days	9	1	Trauma (separation) in pipette	8	3	38%
9 days	11	2 - cyst 1 - degen^{n} 3 - degen^{n}	Trauma Retarded embryo Adverse storage	5	4	80%
8 days	16	1 - cyst	Trauma	15	0	0%



a) oral epithelium.

b) external enamel epithelium.

c) stellate reticulum.

d) stratum intermedium.

e) preameloblasts.

f) odontoblasts.

g) dental papilla.

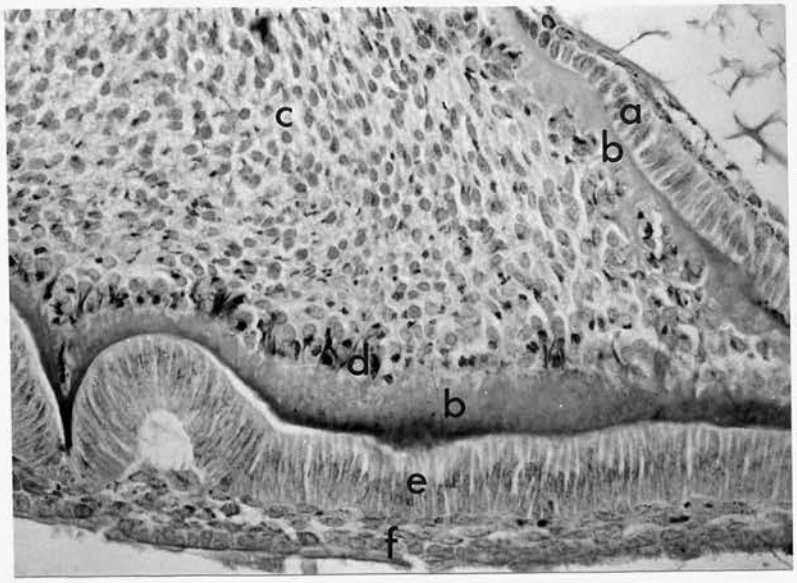
H. & E. x50

Fig. 47. I4 day embryo. M_1 cultured - I2 days.

a) predentine.

Azan x50.

Fig. 48. I4 day embryo M_1 cultured - I2 days.



a) internal enamel epithelium. (lingual wall).

b) predentine.

c) dental papilla.

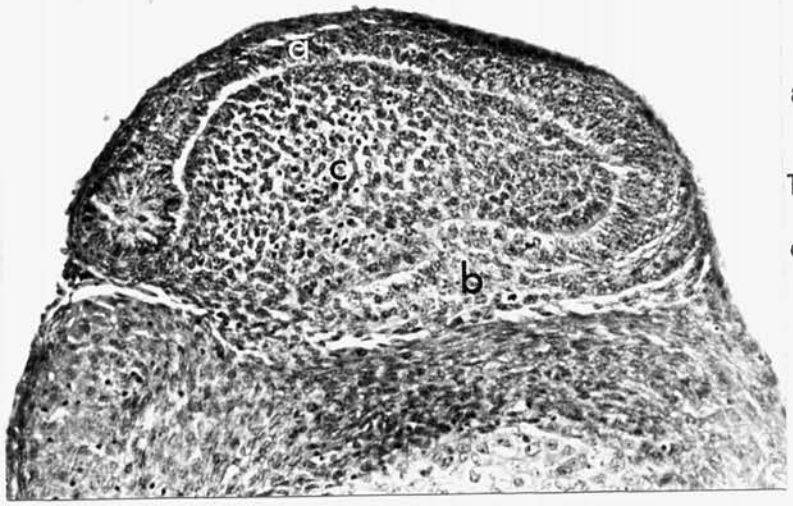
d) odontoblasts.

e) preameloblasts.

f) enamel organ. (labial wall).

Mod. Elftmann technique xI00.

Fig. 49. I3 day embryo.
Incisor cultured I4 days.



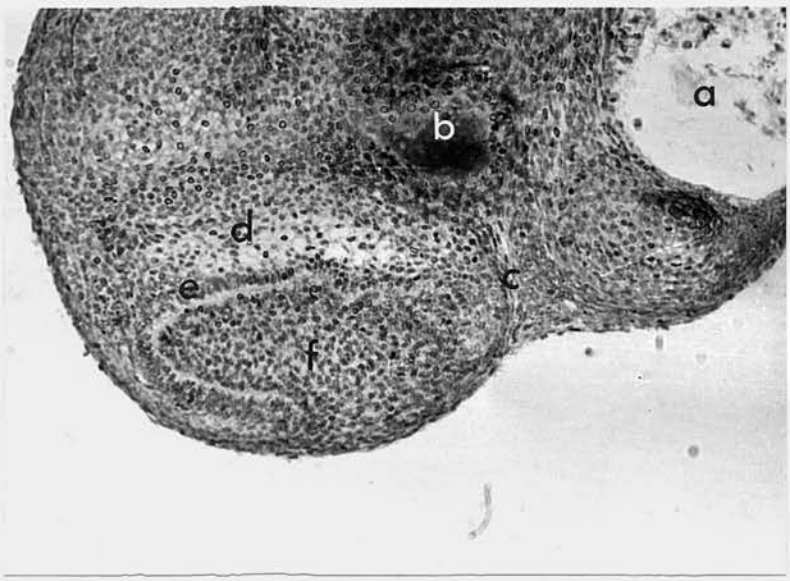
a) internal enamel epithelium.

b) enamel organ.

c) dental papilla.

Allochrome & diastase x63.

Fig. 50. I2 day embryo.
M cultured I5 days.



a) cyst.

b) oral epithelium.

c) external enamel epithelium.

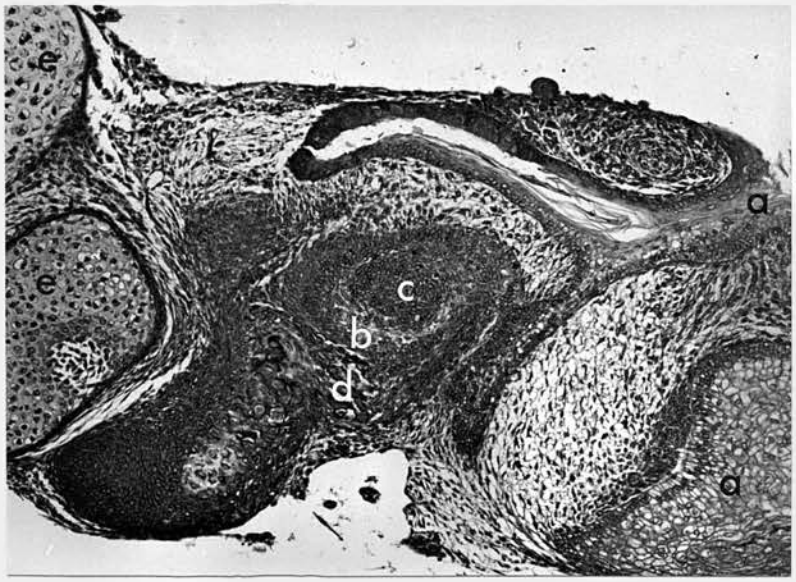
d) stellate reticulum.

e) internal enamel epithelium.

f) dental papilla.

Azan x50.

Fig. 51. II day embryo.
Mandible cultured I4 days : M_1.



a) oral epithelium.

b) enamel organ of tooth germ.

c) dental papilla of tooth germ.

d) osteoid tissue.

e) cartilage.

Allochrome x40.

Fig. 52. I0 day embryo.
Mandible cultured 9 days.

a) keratin.

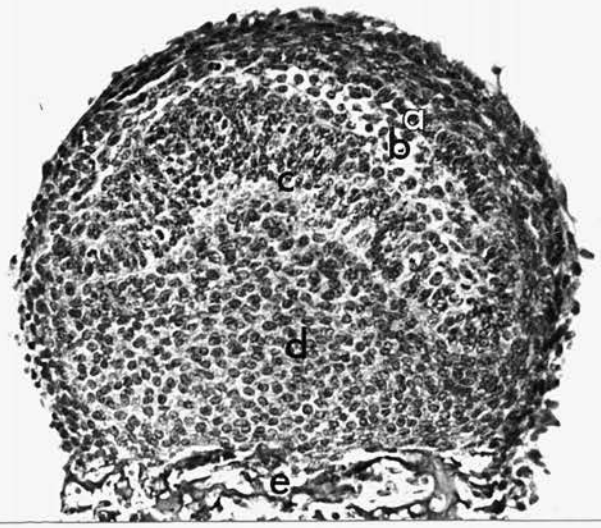
b) preameloblasts.

c) predentine and odontoblasts.

d) dental papilla.

H. & E. x50.

Fig. 53. IO day embryo.
Mandible cultured 9 days : incisor.



a) external enamel epithelium.

b) stellate reticulum.

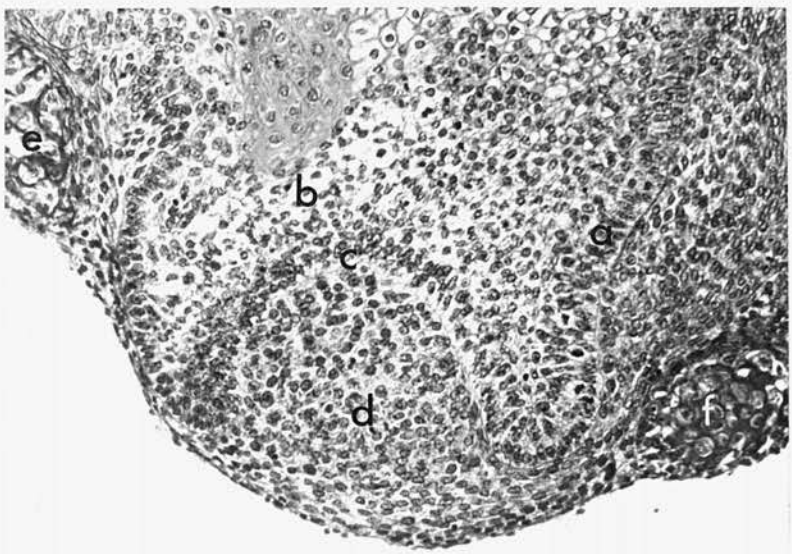
c) internal enamel epithelium.

d) dental papilla.

e) osteoid tissue.

Allochrome & diastase x63.

Fig. 54. 9 day embryo.
Mandible cultured 8 days.



a) external enamel epithelium.

b) stellate reticulum.

c) internal enamel epithelium.

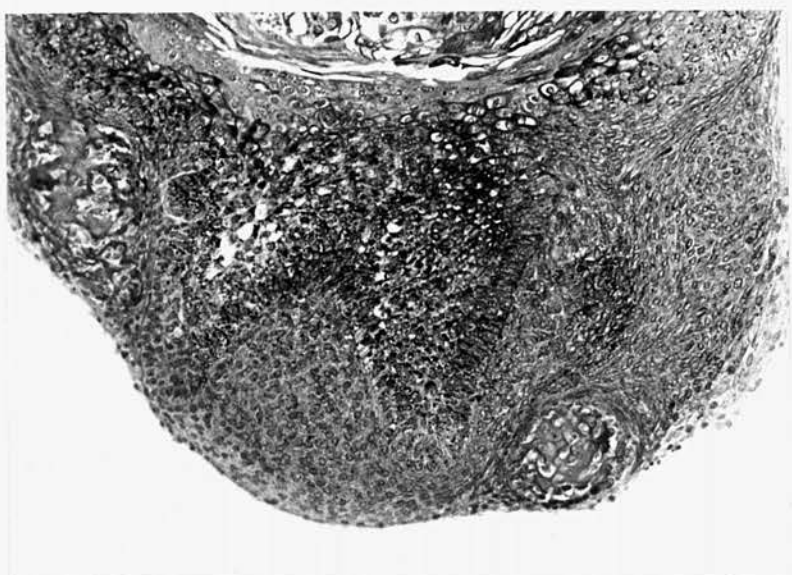
d) dental papilla.

e) osteoid tissue.

f) cartilage.

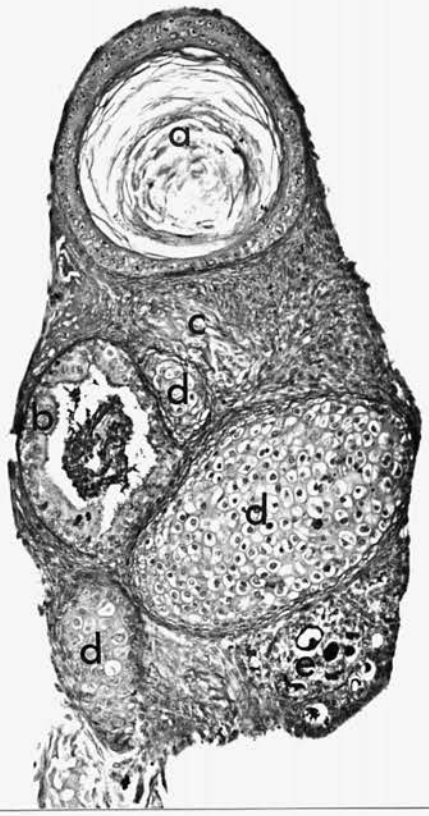
Allochrome & diastase. x50.

Fig. 55. 9 day embryo.
Mandible cultured IO days.



Allochrome x50.

Fig. 56. 9 day embryo.
Same tooth germ : glycogen distribution.



a) epithelial pearl.

b) respiratory type epithelium.

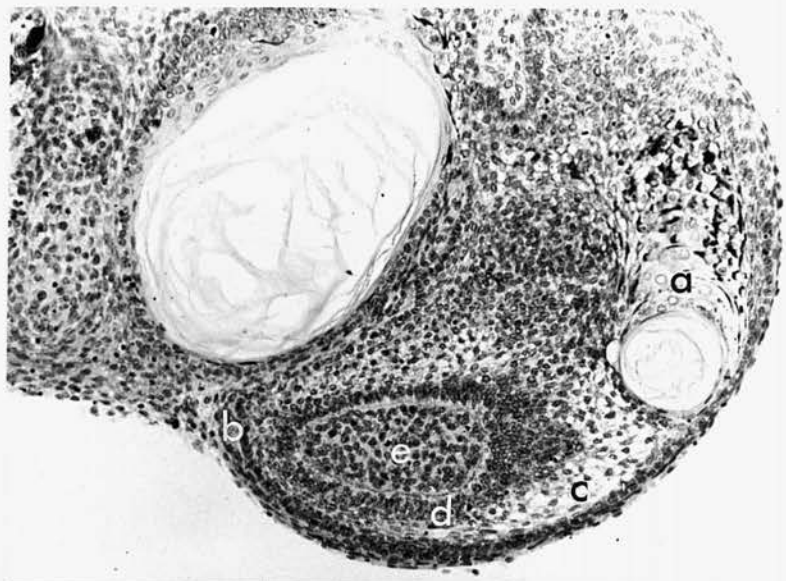
c) osteoid tissue.

d) cartilage.

e) thyroid gland.

Allochrome & diastase x50.

Fig. 57. 8 day embryo.
Mandible cultured I9 days.



a) oral epithelium.

b) external enamel epithelium.

c) stellate reticulum.

d) internal enamel epithelium.

e) dental papilla.

Allochrome x40.

Fig. 58. 8 day embryo.
Mandible cultured I9 days :
tooth germ.

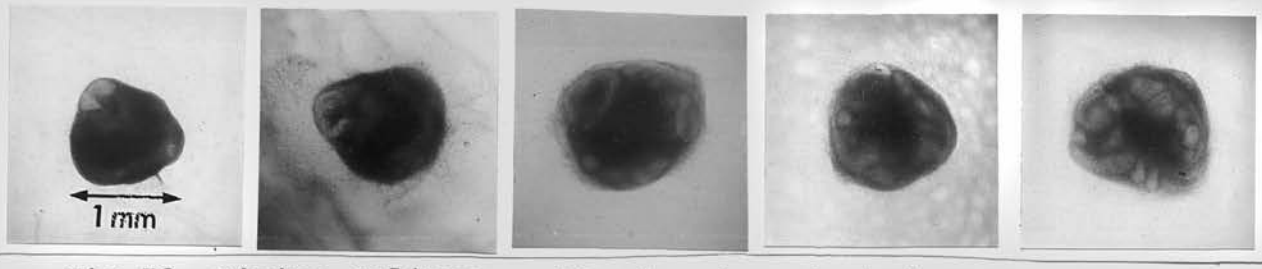
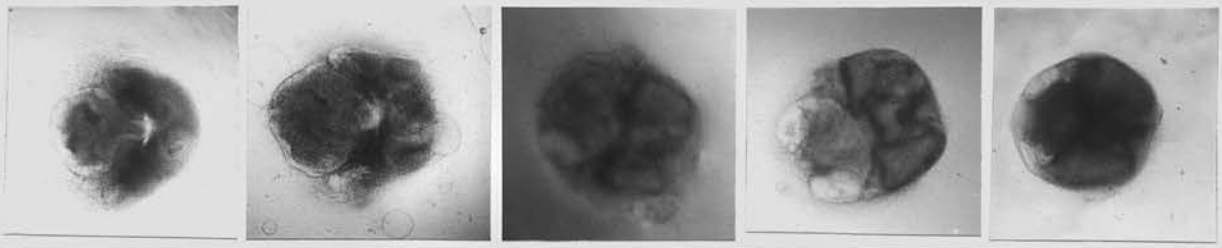


Fig. 59. Living Cultures : Hanging-drop technique.
8 day embryo : Mandibular and Hyoid Arch and
I9 days culture. Rhombencephalon.

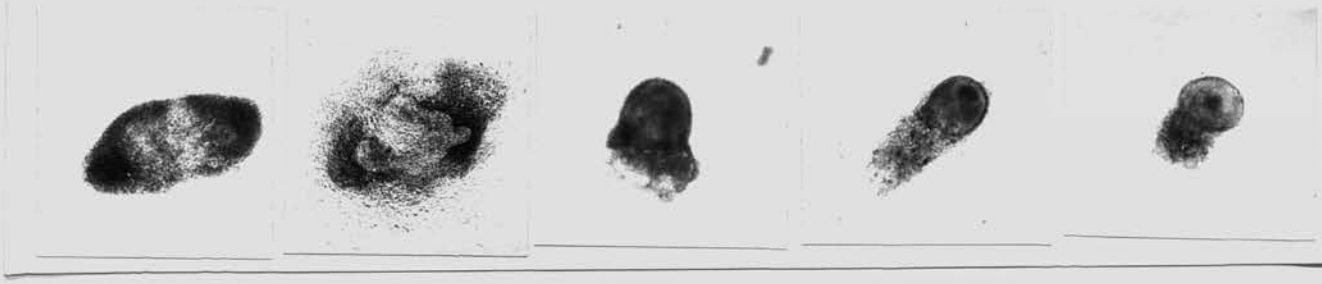
Magnification as above

Fig. 60. Living Cultures : Hanging- drop technique.
8 day embryo : Mandibular and Hyoid Arch.
I9 days culture.



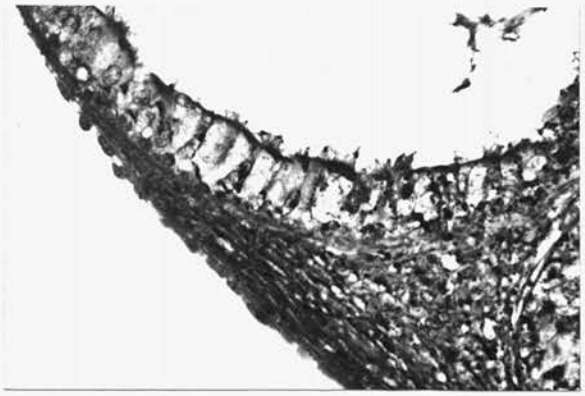
Magnification as above.

Fig. 6I. Living Cultures. : Hanging-drop technique.
8 day embryo : Mandibular and Hyoid Arch and
I9 days culture. Rhombencephalon.



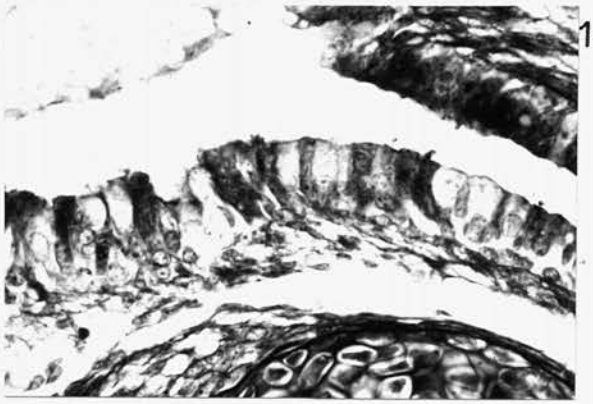
Magnification as above.

Fig. 62. Living Cultures. : Hanging-drop technique.
8 day embryo : Mandibular Arches.
I2 days culture : Degeneration - result of trauma.



Allochrome xI60.

Fig. 63a. Respiratory epithelium in vitro. (8 day at expl'n).

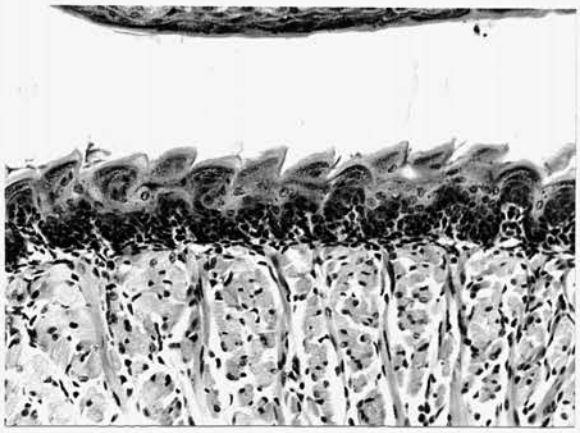


Allochrome xI60.

Fig. 63b. Respiratory epithelium in vivo.

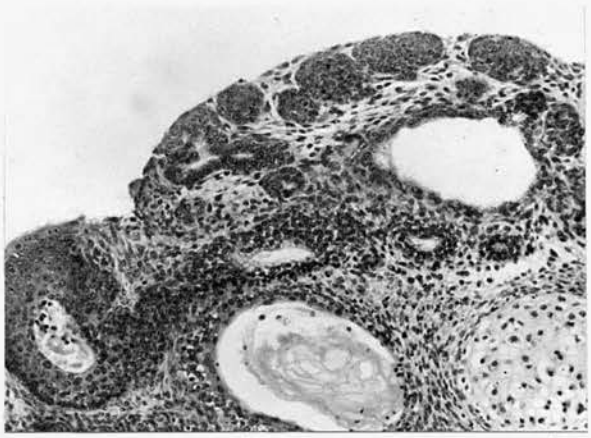
H. & E. xI00.

Fig. 64a. Tongue papillae in vitro. (9day at expl'n)



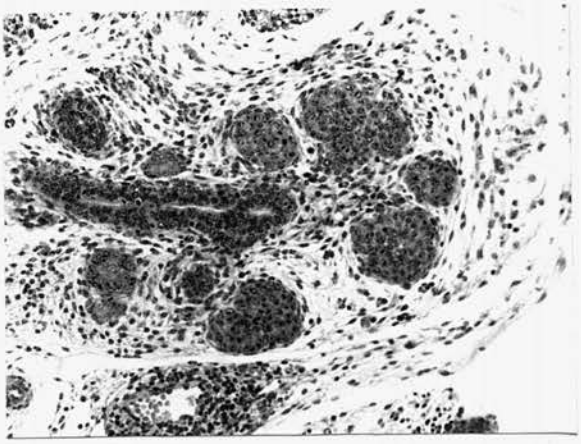
H. & PAS xI00.

Fig. 64b. Tongue papillae in vivo.



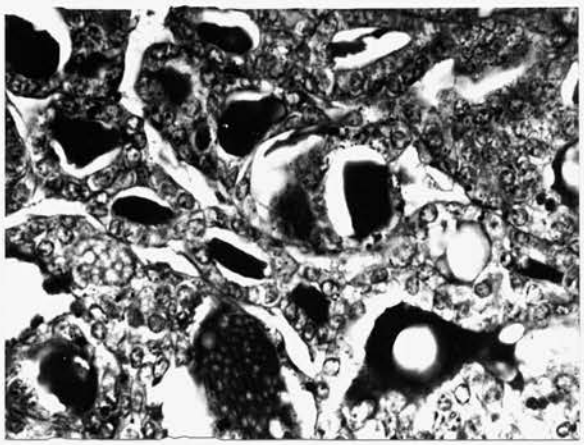
H. & E. x50.

Fig. 65a. Submandibular gland in vitro (8day at expl'n)



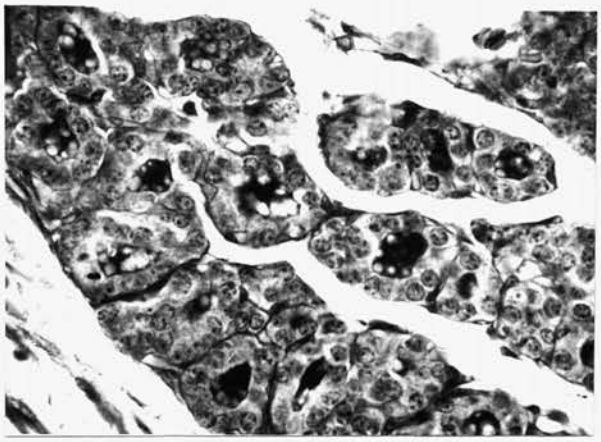
H. & E. xI00.

Fig. 65b. Submandibular gland in vivo.



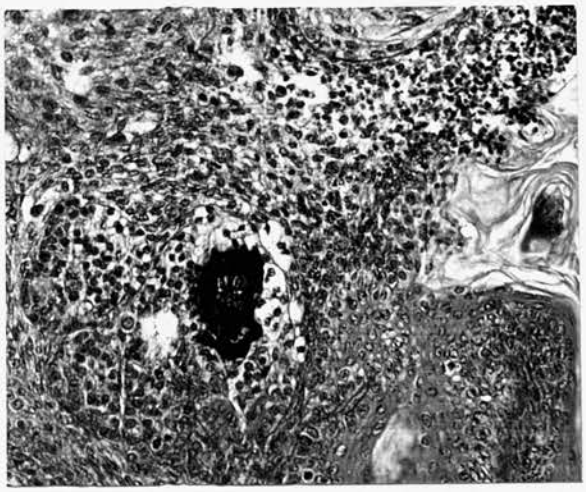
Allochrome x250.

Fig. 66a. Thyroid gland in vitro.
(8 day at expl'n).



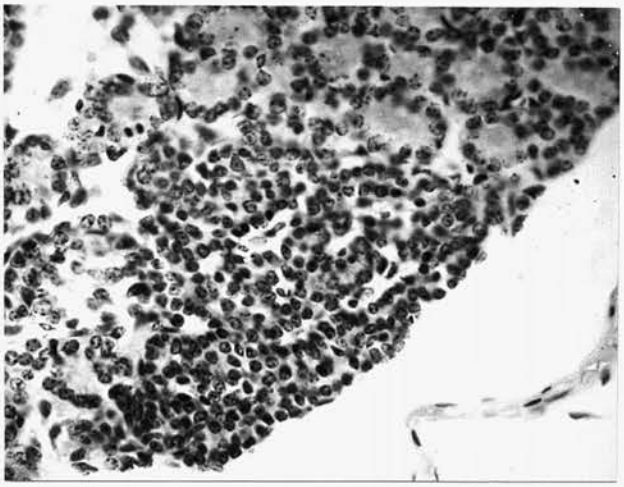
Allochrome xI60.

Fig. 66b. Thyroid gland in vivo.



Allochrome & diastase x63.

Fig. 67a. Parathyroid gland in vitro.
(8 day at expl'n).



H. & E. xI00.

Fig. 67b. Parathyroid gland in vivo.

Figure 68.

DEVELOPMENT OF ODONTOGENIC MANDIBULAR TISSUE - IN VITRO.

x : glycogen.

o : neutral mucopolysaccharide.

• : acid mucopolysaccharide.

DEVELOPMENT OF ODONTOGENIC MANDIBULAR TISSUE - IN VITRO

DISTRIBUTION OF GLYCOGEN, ACID AND NEUTRAL-MUCOPOLYSACCHARIDES

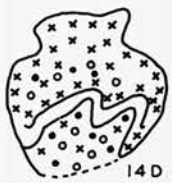
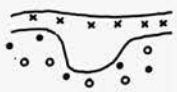
AGE AT EXPL'TN	APPEARANCE OF TISSUES AT EXPLANTATION	APPEARANCE OF TISSUES AFTER CULTIVATION	APPEARANCE OF NEAREST EQUIVALENT IN VIVO STAGE
14 D		 12 D CULTURE	 19 D BIRTH
13 D		 14 D CULTURE	 19 D BIRTH
12 D		A)  6 D CULTURE B)  15 D CULTURE	 15 D  16 D
11 D		 14 D CULTURE	 17 D
10 D		 9 D CULTURE	 15 D
9 D		 10 D CULTURE	 16 D OR 17 D
8 D		 19 D CULTURE	?

Fig. 68.

PART III

DEVELOPMENTAL POTENCIES *IN VITRO* OF ODONTOGENIC EPITHELIUM AND MESENCHYME, SEPARATED AND RECOMBINED.

INTRODUCTION

The progressive manner in which determination for tooth formation is acquired by the mouse mandibular tissues, has been described in Part II.

The limitations on the investigation imposed by the organ culture technique, however, did not allow molar or incisor primordia to be isolated at the time of explantation from very young embryos, and consequently our knowledge is limited to the development of diffuse regions. Thus we know that whole mandibles of 10-day old embryos are determined for the formation of teeth with a molar or incisor morphology. Likewise it seems established that the distal region of the mandible of an 11-day old embryo is determined for incisor formation, the proximal region for molar formation.

Butler (1939) suggested that tooth germs become determined in different ways according to their position in a morphogenetic field within the mandible - a hypothesis further discussed by Van Valen (1962) and Quinet (1964). Finding incisor and molar tooth germs similarly positioned in mandibles developing in vivo and in vitro, Glasstone (1965) interpreted her results as experimental proof of the existence of a morphogenetic field for tooth formation - established in the mouse mandible at the 11-12 day stage. The results with the EZL strain of mouse are subject to the same interpretation except that the field appears to be determined at the 10-day stage.

The establishment of a localized organ field, however, according to Weiss (1939) antedates the fixing of a definitive course of development for the individual cells within the field. Numerous recent reports (see Grobstein, 1967) have shown that few structures in the body/

body are elaborated without an initial interaction of their constituent tissues, and that once initiated, their development continues by a series of sequentially related interactions, i.e. an epigenetic type of development within the organ (Waddington, 1962).

Investigations of the initial interactions of the epithelium and mesenchyme which compose the tooth germ - the sub-units in the mandibular morphogenetic field - have largely been ignored, but the sequential interactions of these tissues in later tooth development have been a basis of study throughout the last century.

The observations of von Brunn (1887, 1891) and several other workers in the field of comparative anatomy, suggested that the epithelial and mesenchymal components of any tooth germ are dependent on each other for their continuing differentiation. von Brunn (1887) postulated that the epithelial sheath is responsible for the development of odontoblasts and dentine, and that its function is primarily that of determining the form of the future tooth. Experimental investigation of the hypothesis in the first half of this century, could only be attempted by mechanical separation of the epithelial and mesenchyme components of the tooth germ followed by transplantation or explantation in culture as isolated or recombined tissue.

Results repeatedly showed that the enamel organ was dependent on the presence of the dental papilla for its continued differentiation. In transplants (Legros & Magitot, 1874; Huggins, McCarroll & Dahlberg, 1934; Hahn, 1941; Lefkowitz, Shapiro & Bodecker, 1947; Zussman, 1966a, b) and <u>in vitro</u> (Glasstone, 1936, 1965; Niizima, 1956; Zussman & Joachim, 1964b) the enamel organs either differentiated to a stratified squamous type of epithelium, formed cysts or degenerated completely. No formation of enamel was seen to take place.

With/

With isolated dental papillae, the cells remained viable, but without the presence of ameloblasts no odontoblasts were formed (Huggins, et al. 1934; Glasstone, 1936, 1965; Lefkowitz, Bodecker & Shapiro, 1944; Zussman & Ioachim, 1946a, b; Zussman, 1966b).

After differentiation, the odontoblasts were no longer dependent on the ameloblasts for their continued development, as dentine was formed both in transplants and in vitro after isolation of the papilla at this stage (Legros & Magitot, 1874; Huggins et al. 1934; Glasstone, 1936, 1965; Hahn, 1941; Szabo, 1954; Niizima & Cattoni, 1958; Nagai & Yoshioka, 1962).

Although the enamel organ seemed to induce odontoblast formation in the underlying cells of the dental papilla, it was found to be incapable of inducing them when recombined elsewhere, e.g. in the central cells of the dental papilla (Huggins et al. 1934; Glasstone, 1965), and in stromal cells of ovaries into which they were transplanted (Hahn, 1941).

Glasstone (1936) assumed that the enamel organ was responsible for the morphological shape of the tooth germ as in its absence, only disorganized bands of dentine were formed instead of the normal regular sheets. Once calcification of the dentine had begun, however, the pulp was then found to be independent of the enamel organ for maintenance of its shape (Hahn, 1941). Gerstner & Butcher (1958) could not agree with the hypothesis of von Brunn that the enamel organ was entirely responsible for the shape of the tooth. On culturing strips of incisor pulp, they found that labial strips grew faster than lingual strips - a result which suggested that the dental papilla itself had an inherent growth pattern.

The interpretations of experiments described above, assumed that the properties of isolated tissues were being examined. Although no evidence to the contrary was reported from the histological findings, most authors noted that the initial/

initial dissection of the epithelium and mesenchyme did not ensure complete separation of the tissues in all cases. Borghese (1950b), examining the development of salivary gland in vitro, found that the epithelial bud, dissected free of most of its mesenchymal capsule, became surrounded during the experimental period by a large compact mass of connective tissue, apparently reconstituted from the remains of the original capsular rudiment. Any similar activity of adhering mesenchyme or epithelial cells in the "separated" tissues of the tooth germ, would invalidate the results described above as being from isolated tissues.

In recent years, however, techniques have been devised which give us a means of reassessing the validity of the results from the early experiments.

In 1952 the Mosconas, following a report by Medawar (1941) on the use of trypsin for preparing pure epithelial sheets, found that the action of the enzyme under controlled conditions, was capable of dissolving the intercellular material of a tissue, and of freeing the constituent cells, which remained viable. On culture, they reconstituted the tissue and differentiated like cultured, but non-trypsinized cells. There followed numerous reports of similar experiments, including a refinement in which the tissues were cleanly separated as layers at the basement membrane (e.g. Zwilling, 1955; Auerbach & Grobstein, 1958; McLoughlin, 1961a, b, and many others). Such methods have been applied in recent years to the study of tooth formation.

These reports, in almost all cases, have confirmed the earlier findings.

With the improved separation techniques, the problems approached were once more those suggested by the hypothesis of von Brunn (1887).

The/

The isolated enamel organ was again found to differentiate only to a squamous type of epithelium *in vitro* (Koch, 1967) and on the chicken chorio-allantoic membrane (Slavkin & Bavetta, 1968a).

The isolated dental papilla, formed no odontoblasts in the cultures of Koch (1967) and Slavkin & Bavetta (1968a), but Pourtois (1966) did identify them in similar cultures, by their cytoplasmic volume, long pseudopodial extensions, and cytochemical characteristics. His conclusions, however, were not at variance with those of the authors of earlier papers where odontoblasts did not form, for he conceded that any odontoblast developing in culture was already determined for such formation at the time of explantation, and therefore may have depended initially on the enamel organ.

The mutual dependence of the epithelial and mesenchymal components of the tooth germ seemed confirmed by all reports : after recombination and culture of these tissues, ameloblasts and odontoblasts differentiated.

The development of the mouse tooth germs cultured by Koch (1967) reached a more advanced stage of differentiation in culture than those of the rat used by Pourtois (1966) and the rabbit used by Slavkin & Bavetta (1968a). Koch's experiments, where the tissues were recombined directly, or separated by a Millipore filter, have shown that the advanced stages of differentiation, not seen before in culture, are remarkably similar to changes *in vivo*. Not only did ameloblasts and odontoblasts differentiate in the usual sequence seen *in vivo*, but their alignment against the Millipore filter mimicked their usual attitude to the basement membrane - normal to the membrane in the case of the ameloblasts, at an angle to it in the case of the odontoblasts. Hard tissue matrices were produced by each tissue, but whereas the predentine matrix formed within as well as on the filter, the matrix formed by the ameloblasts remained on the surface of the membrane. With the deposition of the matrix, the ameloblasts shortened as *in vivo*.

On/

On direct recombination of the enamel organ and dental papilla, calcification of both matrices took place, but when the filter was interposed, only the dentine matrix calcified.

Most recent reports were unable to confirm the theory of von Brunn (1887) and Glasstone (1936) that the enamel organ is responsible for the morphological stage of the tooth germ. In none of the experiments of Pourtois (1966), Koch (1967) and Slavkin & Bavetta (1968a), was tooth morphology restored. Pourtois thought that the role of the enamel organ in controlling tooth morphology was simply that of providing a suitable surface against which the differentiating odontoblasts could align themselves; Koch (1967) assumed it to have a more active role, as odontoblasts did not align themselves experimentally against a similar surface - that of a collagen gel. Although he found collagen not to be a suitable surface for the adhesion of the odontoblasts, he did find that in some way it was concerned with control of tooth morphology (1968). With its removal from the tooth germ by collagenase, normal morphology was distorted in culture within two days, followed by eventual intermixing of all tissues. He concluded - as did Grobstein and Cohen (1965), making a similar observation on developing salivary gland - that the collagen played a role in influencing the epithelial contour.

Kollar & Baird (1969) have carried out experiments with separated and recombined enamel organ and dental papilla specifically to investigate whether the control of morphological structure of the tooth resides in the epithelium, the mesenchyme, or in both. Using as criteria for identification of molar or incisor form, the generalized shape of the tooth germ, and the extent of cyto-differentiation along the length of the enamel organ, they concluded that in the mouse embryo of 13-16 days gestation age, the "shape of the tooth germ is a function of the source of the dental papilla".

Nothing/

Nothing, however, is known about the initial interactions of the odontogenic tissues within the morphogenetic field. To investigate such reactions in the mandibles of 8-12 day old mouse embryos, I designed experiments to find out:-

a) if separation of epithelium and mesenchyme from embryonic mandibles at these stages is possible,

b) if isolated mandibular epithelium and mesenchyme would develop in culture,

c) if tooth germs would develop in culture on recombination of the mandibular epithelium and mesenchyme,

d) whether, if recombination of the tissues allows tooth formation to proceed, it is possible to detect if epithelium or mesenchyme plays a dominant role in controlling future tooth morphology.

METHODS AND MATERIALS

Preparations and procedures where appropriate were carried out aseptically as in Part II.

1. Initial preparation of the explants.

Individual embryos of 8-12 days gestation age were dissected out as in the previous experiments. Retarded embryos were discarded, one litter mate was fixed in Zenker's fluid as a control for each experiment, and all others were transferred to horse serum : Tyrode's solution (1:20 $^{v}/_{v}$). The mandibles were then isolated and the lower surface epithelium removed and discarded from those of 9-12 day old embryos.

2. Separation of epithelium from mesenchyme.

Preliminary experiments showed that separation with crude trypsin (0.5 - 3% for 1-4 hours), released so much mucoid material that further manipulation of the liberated epithelial layer was impossible. The action of the chelating agent Versene (di-sodium ethylene diamine tetra-acetic acid, dihydrate) at a concentration of 0.02 - 0.1% for 1-12 hours, caused visibly incomplete separation, and crude pancreatin (1% for 4-12 hours) gave a clean separation, but the cells did not survive in culture.

The separating medium found to be most effective was that first described by Auerbach & Grobstein (1958), a trypsin-pancreatin solution (3:1 $^{w}/_{w}$) in calcium-and-magnesium free Tyrode's solution. Separation was clean, with little release of mucoid material and viability on subsequent culture was good.

The schedule used was as follows:

a) Individual mandibles were transferred to depression slides, and washed in three changes of calcium-and-magnesium-free Tyrode's solution, the depletion of divalent cations being known/

known to enhance later dissociation by tryptic enzymes (Moscona, 1952).

b) The saline solution was then replaced by the separating medium containing 2.25% crude trypsin (Difco 1:250) and 0.75% crude pancreatin (B.D.H.) dissolved in calcium and magnesium-free Tyrode's solution and sterilized by membrane filtration. Separation was allowed to take place at 4°C., the time required varying with the age of the embryo, being about 15 minutes for the youngest and 3 hours for the oldest.

c) When separation of the epithelial layer from the underlying mesenchyme was seen to have taken place and tested by lifting off part of the layer at the periphery, further activity of the enzymes was inhibited by replacing the solution with another composed of horse serum : Tyrode's solution (1:1 $^{v}/_{v}$). The separation of the tissues was then completed in a solution containing either horse serum : Tyrode's solution (1:20 $^{v}/_{v}$) or, more usually, clot exudate prepared from incubated clots composed of chicken plasma : embryo extract (3:2 $^{v}/_{v}$). Separation was carried out under a binocular dissecting microscope using glass needles which had been drawn out as finely as possible, then broken obliquely to give a yet finer tip.

When the epithelial layer was to be transferred to mesenchyme other than its own, and required more manipulation than usual, a few sterile carbon granules were scattered on the upper surface of the epithelium before complete separation (McLoughlin, 1959). The granules adhered to the surface and made its identification in subsequent handling much easier.

With each experiment, one example of the separated tissues was fixed in Zenker's fluid to confirm adequate separation of the tissues under the particular conditions.

3/

3. Culture of isolated odontogenic epithelium on collagen gel.

To investigate the potential for differentiation in culture possessed by odontogenic epithelium separated from its underlying mesenchyme, oral epithelium was isolated and placed on rafts coated with collagen gel (adult rat-tail tendon collagen, supplied by Dr. J.W. Dodson, then at the Strangeways Research Laboratory, Cambridge). Isolated epithelium has been shown to differentiate to a greater extent when placed on collagen rather than directly on a plasma clot (Dodson, 1963, 1964, 1967). Similar treatment was hoped to encourage differentiation in culture in the present experiments.

4. Culture of isolated mandibular mesenchyme.

To investigate the ability of isolated odontogenic mesenchyme to differentiate, mandibular mesenchyme, separated from oral epithelium, was explanted on Dicel Poult rafts.

5. Recombination of the tissues.

i) Direct recombination of epithelium with its own mesenchyme.

Rafts were used to support the recombined tissues. In the preliminary experiments the rafts were of cellulose-acetate as described by Shaffer (1956), but the mesh was found to be too coarse for satisfactory support of the smaller explants. In later experiments, bright Dicel Poult fabric* (undyed and unsized) was found to be more suitable for such rafts, with one or two strands removed to loosen the weave.

When recombining the tissues, the sterile raft, approximately 4 x 4 mm., was slipped beneath the mesenchyme in the clot exudate. The mesenchyme was positioned on the raft, then the epithelium on the mesenchyme. The fluid was very carefully and slowly withdrawn through a fine pipette from beneath the raft, pulling the epithelium by/

*(supplied by courtesy of Courtaulds Ltd., 22 Hanover Square, London, W.1.)

by surface tension down on to the mesenchyme. The dissecting needle was used to keep the epithelium in position as much as possible, and its progress was watched by keeping the epithelium in focus in the dissecting microscope. Before placing each raft on the prepared plasma clot for culture, it was briefly touched on dry surfaces of the sterile depression slide to remove excess solution by capillary action.

It was shown in Part II of the thesis, that fragments of mandibles from 8-10 day old embryos would not develop in culture, and although fragments of mandibles from 11 day embryos would do so, the survival rate of these explants was very low. To investigate control of tooth germ differentiation, it is obviously desirable to isolate the individual primordia, and culture recombinations of such epithelial and mesenchymal components of tooth germs isolated from the primordia of other mandibular structures - as was achieved in the experiments of Pourtois (1966), Koch (1967) and Slavkin & Bavetta (1968a), who worked with odontogenic tissues of older embryos. However, it is equally obvious that if small fragments of very young mandibles are incapable of differentiation in culture, fragments further traumatised by the separation technique would have very little chance of survival in vitro. It was hoped to overcome this limitation by manipulating, not the isolated incisor or molar primordia, but the complete sheet of oral epithelium covering the mandibular arch, repositioning the tissues on recombination so that the tooth germ primordia were again brought into contact.

Knowing that in some instances, trypsin activity is capable of destroying inductive activity of tissues (Hayashi, 1958; Saxén & Toivonen, 1962), it was thought advisable to investigate the effect of the present experimental procedures on the odontogenic tissues, by recombining the tissues with the epithelium and mesenchyme re-united exactly as in vivo, before altering the orientation experimentally.

ii/

DIAGRAM OF RECOMBINATION OF MANDIBULAR EPITHELIUM AND MESENCHYME
WITH
INCISOR EPITHELIUM COMBINED WITH MOLAR MESENCHYME
MOLAR EPITHELIUM COMBINED WITH INCISOR MESENCHYME

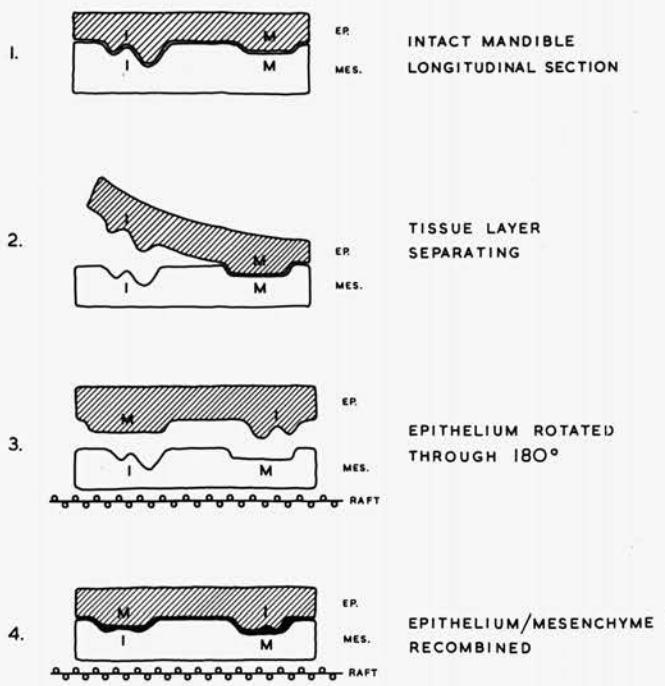


Fig. 69.

ii) Recombination of transposed epithelial and mesenchymal components of molar and incisor tooth germs.

By separating the oral epithelium from the mandibular mesenchyme, and rotating it through 180° so that on recombination, incisor epithelium overlay molar mesenchyme, and molar epithelium overlay incisor mesenchyme (Fig. 69), it was hoped to find that:-

a) tooth germs would develop with a morphology dictated by the 'dominant' tissue controlling tooth form at the time of isolation, i.e. if at explantation, the epithelium were evocating mesenchyme to conform to the epithelial morphological pattern, then incisiform morphology should result from the combination of incisor epithelium and molar mesenchyme; if mesenchyme were evocating the overlying epithelium to conform to the mesenchymal morphological pattern, then the same combination of incisor epithelium and molar mesenchyme would result, instead, in molariform morphology, and that

b) tooth germs would develop such that incisiform and molariform morphology would be present in the same explant, thus enabling comparisons to be made of development of the different tooth forms under identical conditions.

It was hoped that by identifying the morphology formed by the recombined tissues during culture, it would be possible to deduce whether epithelium or mesenchyme was responsible for dictating future tooth form at the time of isolation.

iii) Recombination of oral epithelium and mandibular mesenchyme from different ages of embryo.

The results of Part II of the thesis, showed that an analysis of tooth form developing in culture, enabled us to identify the main stages of determination for tooth formation in the odontogenic regions: no determination (8-day stage); determination for cuspless teeth (9-day stage); determination for teeth of typical morphology (10-day-and-older stage).

It/

It was hoped that by separating the odontogenic regions at these stages into epithelial and mesenchymal components, and recombining each with the opposing tissue of another stage, tooth germs would develop in culture. An analysis of the tooth form developing from the recombination, would enable us to identify the state of competence or of active evocation of the individual tissues (rather than the regions), at the time of recombination and explantation. Such results would enhance any results found by culture of such recombinations as described in ii) above.

6. Method of Cultivation.

All explants were cultured by the Watch glass technique as described in Part II, except for those tissues from 8-day old embryos which were cultured by the hanging-drop technique. Large explants were cultured in an atmosphere of approximately 58% oxygen and 3% carbon dioxide in air, as in previous experiments.

7. Subculture of explants.

Subculture was carried out every second day. No dissection of the explants was attempted : the rafts were dissected free from the old plasma clots, and placed on freshly prepared ones.

8. Histological treatment.

Histological treatment was as outlined in Parts I and II, with only a few minor modifications:-

i) the fixative was allowed to infiltrate into the explant in situ, before the raft was dissected free and transferred to the bulk of fluid for further treatment,

ii) after dehydration, and before embedding, the raft was removed by soaking the explant in acetone for ½-1 hour. Dicel Poult was found to dissolve as easily as the cellulose acetate netting.

The/

The tissues were sectioned at 5 μ , and as before, consecutive slides were stained with Haematoxylin and Eosin, Allochrome, Allochrome and diastase, and the Alcian blue method.

9. Methods of examination.

Methods of examination were as for Part II.

RESULTS

1. Separation of oral epithelium from mandibular mesenchyme.

As can be seen from Figs. 70 to 74, trypsin-pancreatin (2.25 : 0.75% $^{w}/_{w}$) in calcium and magnesium-free Tyrode's solution, cleanly separated oral epithelium from the underlying mesenchyme at all ages investigated. The times taken by the tissues to separate, varied with the age of the embryo, being about 15 minutes at 4°C. for 8-day old embryos and 3 hours for the 12-day old embryos. The epithelial layer peeled easily off the mesenchymal tissues, and histological treatment showed that the cells looked healthy, mitotic figures were present (Figs. 73, 74), and the histochemical pattern seemed undisturbed. The basement membrane was completely absent. The epithelium, once separated, curled up with the basal side innermost. Although it peeled cleanly off the mesenchyme from the 8-, 9- and 10-day old embryos, subsequent manipulation of the very small sheets of tissue without damaging them was very difficult.

2. Culture of isolated oral epithelium on collagen gels.

Oral epithelium was isolated from its underlying mesenchyme from 5 embryos at 11-days gestation age, and cultured by the Watch glass technique on collagen gels, for 2, 4, 6, 10 and 12 days. None of the explants appeared healthy at the end of their period in culture. Most of them showed only a mass of degenerate cells, but even where the epithelium could still be seen as a layered structure, 4 or 5 cells thick (Fig. 75) the cells were rounded up retracted from their neighbours, and had indistinct nuclear detail.

3/

3. Culture of isolated mandibular mesenchyme.

Mandibular mesenchyme was isolated from its oral epithelium from 4 embryos of 11-days gestation age, and cultured by the Watch glass technique for 4, 6, 10 and 14 days.

The tissues survived in all cases, and differentiation took place. Throughout the range of times in culture, all stages of chondrogenesis could be seen, from the whorl of condensed cells at the elongating tip of the rod of cartilage, to the fully formed tissue showing intercellular matrix and cells, with the typical histological and histochemical features of Meckel's cartilage *in vivo* (Part I) and *in vitro* (Part II) Fig. 76.

Osteoid tissue formed around the cartilage. Its early differentiation was seen after 4 days in culture, the rate of development therefore being about 75% that of its rate *in vivo*. After 10 days in culture, the matrix appeared to be calcifying.

The mesenchymal intercellular material stained positively for acid and neutral mucopolysaccharides as *in vivo*, and fine collagen fibrils were present.

Odontoblasts could not be identified, either as a group of cells aligned against any surface, or as discrete cells with large cytoplasmic volume and long pseudopodial extensions.

Isolated mandibular mesenchyme from an 8-day old embryo, cultured for 8 days by the Hanging drop technique, degenerated.

4. Culture of separated and recombined tissues.

i) Direct recombination of epithelium and its own mesenchyme.

a) Recombination of tissues from 8-day old embryos.

Because of the difficulty of manipulating the very small sheet of mandibular epithelium completely separated from the mesenchyme, the oral surface epithelium was gently lifted off the underlying mesenchyme, leaving it attached anteriorly to the ventral surface epithelium which was left undisturbed. It was then pulled gently/

gently back into position, and the explant cultured by the hanging drop technique. Only one explant was made. After culture for 12 days, the tissues appeared healthy (Fig. 77). The epithelium had differentiated to a keratinising stratified squamous epithelium, showing a large epithelial pearl. The distribution of glycogen within the tissues, was as in the cultured, non-separated epithelium described in Part II. No epithelial derivative - salivary gland, thyroid gland, or tooth germ, had formed.

Cartilage was found to have differentiated within the mesenchyme with the usual histological and histochemical characteristics as described in Part II. Very little osteoid tissue was present, and in this explant was positioned near to the plasma clot. The mesenchyme stained for acid and neutral mucopolysaccharides in the intercellular matrix as *in vivo*, and glycogen granules and young collagen fibrils were present.

b) Recombination of tissues from 9-day old embryos.

3 examples of recombinations of separated mandibular epithelium and mesenchyme were made from 9-day old embryos, and cultured by the Watch glass technique. No outgrowth of fibroblasts was seen from any of the explants, degeneration appearing to start immediately on explantation.

c) Recombination of tissues from 10-day old embryos.

3 examples of recombinations of separated mandibular epithelium and mesenchyme were made from 10-day old embryos. One explant became infected after 6 days and was discarded, the other two explants were fixed after 8 days in culture. Both, on histological examination, showed dead mesenchymal cells, with little evidence of any epithelial cells (Fig. 78).

d) Recombination of tissues from 11-day old embryos.

4 examples of recombinations of separated mandibular epithelium and mesenchyme were made from 11-day old embryos, and cultured by the Watch glass technique for 6, 11 and 14 days. One/

One explant became infected, but in the other three explants, two well-formed molar tooth germs developed (Figs. 79 and 80).

Both tooth germs developed during 11 days in culture, in explants which bore both mandibular arches, and were oxygenated during the culture period.

The tooth germs could be identified unequivocally as molars. One was slightly distorted by the raft, but both were positioned in the usual anatomical position of the molar tooth germ, lateral to the tongue and the developing salivary gland ducts; both developed at right angles to the oral epithelium, and showed a broad presumptive occlusal surface and wide cervical loop in relation to the depth of the walls of the enamel organ. Early cusp formation was seen but distorted (Fig. 80). The dental lamina for the future M_2 was present in both cases.

The histological and histochemical appearances of both were similar to those of the 16-day stage molar in vivo (Fig. 26), giving a rate of development in vitro after recombination of about 45% that of normal. The external enamel epithelia were composed of cuboidal cells staining positively for glycogen in all regions except the cervical loop. The stellate reticula were well differentiated, with cells widely separated by an intercellular matrix that contained sparse glycogen granules and that stained weakly for neutral-, but strongly for acid-mucopolysaccharides. The internal enamel epithelia were composed of columnar cells, most fully differentiated at the antero-lingual walls, where the nuclei had begun to move away from the basement membranes. The cells of this layer contained sparse glycogen granules most obvious in the most fully differentiated cells. The dental papillae contained cells more densely packed than in the surrounding connective tissue, and odontoblasts appeared to be aligning against the basement membrane in the antero-lingual walls. No predentine matrix was present. The dental papillae stained positively near the/

the periphery for acid mucopolysaccharide, and uniformly for neutral mucopolysaccharide. No glycogen could be seen.

In all three surviving explants, the epithelium no longer extended over the complete surface of the mesenchyme, but was confined to a central zone. It had differentiated in particular regions to form cysts and epithelial pearls (particularly where tooth germs could have been expected to develop, Fig. 81), into the papillary pattern on the dorsum of the tongue, and into respiratory type epithelium at the proximal (dorsal) region of the tongue.

Salivary gland ducts had developed medially to both tooth germs (Fig. 83). In one explant, no branching of the main duct was seen, but the other explant showed more advanced differentiation : all ducts were lined with a double row of cuboidal cells which contained PAS-positive staining granules, and the intraglandular ducts were hollow and contained a little PAS-positive stairing material. Approximately eleven adenomeres were present.

In one of the cultures, a large autonomic nerve ganglian was seen, possibly of the parasympathetic nervous system (Hay, 1956).

As for the general development of the mesenchyme; in the present cultures and in all recombinations to be described, the separation procedure appeared to have no effect on the differentiation of cartilage and osteoid tissue. All stages of differentiation of rods of cartilage (Meckel's cartilage), and the surrounding dentary could be seen, depending on the time in culture. With culture of 11- and 12-day stage mandibles for 11-14 days, particularly fine examples of endochondral ossification of the central zones of the rod of cartilage as seen <u>in vivo,</u> could be observed. Osteoid tissue extended towards any developing tooth germ <u>in vitro</u> as described in Part II, and showed calcification in the longer term cultures.

The/

The cartilage and osteoid tissues appeared to have the histological and histochemical features described in Parts I and II. No further descriptions will therefore be given of the development of these tissues in the cultured recombinations, unless specific features are of importance.

e) Recombination of tissues from 12-day old embryos.

4 examples of recombinations of separated mandibular epithelium and mesenchyme were made from 12-day old embryos, and cultured by the Watch glass technique for 10, 12 and 14 days. None of the explants was oxygenated during culture. One explant degenerated, the others appeared healthy after their period in culture, but only one tooth germ developed, fixed after 14 days in culture.

The successfully developed tooth germ had the histological appearances of a 15-day stage molar *in vivo*, but the histochemical pattern of a 16-day stage tooth - a maximum rate of develcpment *in vitro* of about 29% of normal (Figs. 82 and 94).

The tooth germ was in the late cap stage of development, showing little invagination of the enamel organ walls. The external and internal enamel epithelia were composed of cuboidal cells, staining positively for glycogen, The stellate reticulum showed little intercellular material, but appeared to stain for glycogen, and faintly positive for both acid and neutral mucopoly-saccharides. No hard tissue matrix, and no odontoblasts were present. The dental papilla contained more densely packed cells than elsewhere in the mesenchyme and osteoid tissue surrounded the tooth germ as *in vivo*.

The oral epithelium showed the same characteristics of development as in the recombinations of tissues from 11-day old embryos. It was confined to the central zone of the explant, was mostly of the keratinising stratified squamous type, showing numerous cysts, and the papillary pattern of the tongue. A small area of respiratory type epithelium was present dorsally.

Salivary/

Salivary gland development showed well differentiated gland, containing over thirty adenomeres after 12 days in culture (Fig. 84). Mitotic figures could be seen in the walls of the ducts, which were hollow, and lined by a double row of cuboidal cells containing PAS-positive staining granules.

ii) Recombination of transposed epithelial and mesenchymal components of molar and incisor tooth germs.

a) Recombination of transposed tissues from 8-, 9- and 10-day old embryos.

No examples of transposed recombinations of separated mandibular epithelium and mesenchyme were made from 8, 9 and 10 day old embryos. The small sheets of epithelium, though capable of clean separation from the mesenchyme, were found impossible to manipulate into position without damage.

b) Recombination of transposed tissues from 11-day old embryos.

3 examples of recombinations of separated mandibular epithelium and mesenchyme, with the epithelium rotated through 180° before recombination, were made from 11-day old embryos. Two of the explants appeared to be developing well, but became infected after 6 days in culture. The third explant formed healthy tissues during 14 days in culture. One tooth germ developed.

The morphology of the tooth germ did not strictly resemble that of either a typical molar or incisor tooth germ, and was, moreover, slightly distorted by the present of the raft (Figs. 85, 86 and 95). It most resembled an incisor tooth germ in that predentine formation was present at the anterior tip of the tooth and down one wall only. There was no broad presumptive occlusal surface, and only a small mass of stellate reticulum could be seen at the anterior tip of the tooth germ. One wall of the enamel organ was greatly elongated. Less credibly, it could be likened to a molar/

molar form by postulating a sagittal section and distortion by the raft which would respectively emphasise its elongated shape and explain the small area of stellate reticulum.

If likened to an incisor tooth germ, the rate of development in culture was approximately 43% that of normal. If likened to a molar tooth germ, showing predentine formation, then its rate of development in culture was approximately 57% that of normal (a much higher rate than would have been expected by analogy with other cultures). Whether the tooth germ resembled most the incisor or molar tooth germ, its orientation in the culture with respect to the cartilage, bone and the rest of the epithelium was abnormal, and will be discussed later.

The enamel organ was composed of an external enamel epithelium of cuboidal cells, except on the elongated wall, where they appeared to be squamous (as in the incisor *in vivo*): the stellate reticulum was small in volume, but contained intercellular material staining for neutral mucopolysaccharide. No section containing the stellate reticulum was stained by the Alcian blue method. The internal enamel epithelium was composed of columnar cells, most fully differentiated at the anterior tip and the short wall of the tooth germ. All cell layers of the enamel organ contained glycogen granules, except at the anterior tip of the tooth in the internal enamel epithelium. Predentine was present at the tip and short wall of the tooth germ, and odontoblasts were present. The dental papilla was composed of condensed mesenchymal cells, and the intercellular material stained positively for both acid and neutral mucopolysaccharides. Glycogen granules were confined to the connective tissue at the periphery of the dental papilla.

From careful assessment, its position in the explant indicated that it was formed by the recombination of incisor epithelium and molar mesenchyme.

The epithelium again did not extend over the surface of the mesenchyme. It had differentiated to a stratified squamous type of epithelium,/

epithelium, and showed cyst formation where the combination of molar epithelium and incisor mesenchyme could have been expected. No tongue was present, and no papillary pattern or respiratory type epithelium had differentiated. No salivary gland tissue had developed.

The differentiation of the cartilage and bone appeared to be unaffected by the manipulations of the epithelium, and was as described in the previous cultures.

c) Recombination of transposed tissues from 12-day old embryos.

2 examples of recombinations of transposed mandibular epithelium and mesenchyme were made from 12-day old embryos. One explant degenerated, but the other showed very good differentiation of s tructure during 11 days in culture. Two tooth germs formed, one unequivocally incisiform (Figs. 87 and 95), the other molariform (Figs. 88 and 95).

The incisiform tooth germ had the elongated shape of the incisor in vivo, with a narrow incisal tip and cervical loop in relation to the depth of the walls of the enamel organ. One wall of the enamel organ was more fully differentiated throughout its length than the other.

Whenever the external enamel epithelium was adjacent to stellate reticulum it was cuboidal, and elsewhere squamous. The stellate reticulum, present in one wall only of the tooth germ, and at the 'incisal' tip, contained cells widely separated by intercellular material which stained strongly for acid mucopolysaccharide. The cells of the internal enamel epithelium were most columnar at the incisal tip, and gradually decreased in height towards the cervical loop a s in vivo. Glycogen granules were present in all cell layers of the enamel organ, except in the internal enamel epithelium on the less fully differentiated side, as in the incisor in vivo. The dental papilla was composed of densely packed cells.

Pre-/

Pre-odontoblasts appeared to be aligned against the basement membrane at the tip of the tooth. The papilla stained very much more intensely for both neutral and acid mucopolysaccharides than the surrounding connective tissue, and contained fine glycogen granules, but no collagen fibrils.

A very careful identificationof orientation during culture and histological preparation, showed that this incisiform tooth germ developed where incisor epithelium was recombined with molar region mesenchyme.

During the manipulation of the epithelium, the orientation of Meckel's cartilage and surrounding osteoid tissue is undisturbed. However, due to the abnormal position of this tooth germ in the 'dorsal' or proximal region of mandibular mesenchyme, Meckel's cartilage ran a seemingly abnormal course anterior to the incisal tip (Fig. 89). Indeed the osteoid tissue appeared to be actually invading the enamel organ (Fig. 90).

The other tooth germ was molariform (Fig. 88) showing a broad presumptive occlusal surface with cusp formation, and a wide cervical loop in relation to the depth of the walls of the enamel organ. The dental lamina for M_2 seemed to be developing.

The external enamel epithelium was composed of cuboidal cells, the stellate reticulum was well differentiated, and stained strongly positive for acid mucopolysaccharide, weakly for neutral mucopolysaccharide. The internal enamel epithelium was composed of columnar cells, more fully differentiated on one cusp than the other. All cell layers of the enamel organ contained glycogen granules except at the cervical loop. The dental papilla contained densely packed cells, and odontoblasts were present in the most fully developed cusp. As with the incisiform tooth, the papilla stained intensely for acid and neutral mucopolysaccharide, and fine glycogen granules were present. In this tooth germ, however, fine collagen fibrils were present.

In/

In this explant, as with the explant from the 11-day old embryo, where the epithelium was rotated through 180°, the orientation of the tooth germs to each other, and to the other structures in the mandible, was abnormal (Fig. 89) and will be discussed later.

No tongue was present in the explant, no papillary pattern had developed, and no respiratory type epithelium was seen.

Salivary gland showed advanced differentiation. Over thirty adenomeres were present, mitotic figures were much in evidence, and the intraglandular ducts were hollow. Cell debris was present in the main duct. The lining cells of the ducts, contained PAS-positive granules, not removeable with diastase.

iii) Recombination of oral epithelium and mandibular mesenchyme from different ages of embryo.

2 examples of 12-day stage epithelium combined with 9-day stage mesenchyme, and 2 examples of 9-day stage epithelium combined with 12-day stage mesenchyme, developed in culture during 11 and 16 days.

In all 4 explants, the mesenchyme developed similarly. Rods of cartilage (Meckel's cartilage) were present, replaced centrally in the longest term cultures with osteoid tissue by endochondral ossification, and surrounded by the osteoid tissue as *in vivo* by membranous ossification.

The differentiation of the epithelium varied with the source of the tissues.

In both explants containing 9-day stage epithelium, most of the epithelium had formed keratinising epithelial pearls, and had made no attempt to spread over the mesenchyme as a layer of tissue. No tooth germ formation was seen. Salivary gland tissue, however, had developed in both explants, forming isolated glandular inclusions within the mesenchyme, but showing no exit of the main duct into a larger mass of epithelium. After 16 days in culture/

culture, one gland consisted of a branching main duct from which about three adenomeres appeared to be developing (Fig. 92). The ducts were hollow, and lined by a double row of cuboidal cells which contained granules staining positively by the periodic-acid Schiff reaction. After 11 days in culture, the other explant showed a more fully differentiated salivary gland (Fig. 93). About eight adenomeres were present, leading to large dilated main ducts.

With the explants containing 12-day stage epithelium, a different pattern of differentiation was seen. At the time of explantation, the epithelium overlapped completely the 9-day stage mesenchyme. After 11 and 16 days in culture, the epithelium was found to have degenerated to such an extent that it was present only as keratinizing epithelial pearls in one explant. In the other explant, it differentiated to cover a larger area of mesenchyme as a keratinising stratified squamous epithelium, showing projections invaginating into the underlying mesenchyme. One such large projection surrounded a core of mesenchymal cells and could have represented a rudimentary attempt at tooth formation (Fig. 91). No salivary gland development was seen in either of these two explants.

DISCUSSION

In vitro cultivation of the separated and recombined odontogenic tissues from young mice of 8-12 days gestation age, has shown that, in general, the experimental procedures left the tissues relatively unharmed. Tooth formation was able to proceed much as *in vivo* in many cases. There is some evidence for believing, however, that the epithelial cells in particular were not quite as healthy and unaffected by the experimental methods as histological examination of the newly separated tissues suggested them to be.

Consistently found 'shrinkage' of the epithelium on the mesenchyme, and failure of some epithelial derivatives to develop, indicated that cell death in the epithelium may have taken place. Such a reaction on the part of this tissue has been previously noted after similar procedures.

Shizuya (1961) found that enzymatic separation of tissues by trypsin, selectively lowered the respiratory quotient of the epithelial layer of the mucosa, but left the connective tissue relatively unaffected. McLoughlin (1961a, 1968) found that after separation of chick limb bud epidermis from its underlying mesenchyme with 5% crude trypsin, histology showed cleanly separated healthy layers of epithelium similar to the separated odontogenic epithelium in the present experiments. On recombination and culture, however, during the first 24 hours, cell death in the limb bud epithelium was approximately 50%. Recovery was remarkable, and an apparently normal epithelium was quickly reconstituted by phagocytic action of the periderm cells, and reorientation of the surviving basal cells. As no localized derivatives were involved in such experiments, no assessment can be made on the effect on future development of structure after such extensive reorganization.

In/

In the present experiments, massive cell death coupled with reconstitution of the layered structure as described by McLoughlin (1961a, 1968) and a lack of spreading of the epithelium on the mesenchyme - a frequent feature of such recombinations (McLoughlin, 1960a,b, 1961a; Grobstein, 1967) could account for the shrinkage of the epithelium. The presence of respiratory type cells incorporated into the epithelial layer at its dorsal periphery tends to support the theory. Most epithelial cells differentiate according to the mesenchyme with which they are combined (McLoughlin, 1960a,b, 1961a, 1968; Billingham & Silvers, 1968) and epithelium malpositioned by contraction of the reconstituting layer would not be evident. Respiratory epithelium, on the other hand, once determined remains as respiratory epithelium on heterologous mesenchyme (Aydelotte, 1965). In the present experiments, the respiratory epithelium at the dorsal extremity of the epithelial layer was found at an unusually distal (anterior) site, on the dorsum of the tongue at the level of the first molar and submandibular gland duct, a level where keratinising squamous epithelium of papillary lingual pattern normally prevails.

Development of the recombined tissues, also seemed to be affected by oxygenation during the period of culture. Whereas such treatment had no marked influence on either the rate or extent of differentiation of the epithelium in the cultures of mandibular fragments as described in Part II, this was not the case in the present experiments. Those explants oxygenated during culture, showed better development of tooth germs than those cultured in air. It is possible that the factors responsible for the lowering of the respiratory quotient of the epithelium following trypsin separation, as found by Shizuya (1961) responded to the elevated oxygen tension, and the detrimental effects were counteracted to a certain extent.

Very few significant results on the interactions of mandibular tissues from 8-10 day old embryos were found on culture of the separated and recombined tissues. Trauma, and culture on rafts by the/

the Watch glass technique rather than by the hanging drop technique, possibly account for the degeneration of the recombinations of tissues from 9- and 10-day old embryos. The use of the hanging drop technique, and the minimum of trauma, possibly account for the survival in culture of the recombined mesenchyme and epithelium explanted from the 8-day old embryo. The absence of any epithelial derivative in this case seems attributable directly to the separation technique.

The information discovered from the present experiments about epithelio-mesenchymal interactions in the early stages of odontogenesis, has come from recombination and culture of the mandibular tissues of 11- and 12-day old embryo mice.

Neither odontogenic epithelium, nor mesenchyme, developed into tooth structure when cultured in isolation. Their dependence on each other seemed confirmed by their differentiation into tooth germs in culture, when recombined. Such interdependence between the epithelial and mesenchymal tissues had already been demonstrated at later stages of development (Pourtois, 1966; Koch, 1967; Slavkin & Bavetta, 1968a; Kollar & Baird, 1969).

The tooth germs of the EZL strain of mouse developing in culture from the recombined tissues, showed no distortions attributable to the separation technique. The morphological and histological development of the molar tooth germs were as similar to the normal tooth germs *in vivo*, as were those developing in culture from tissues of similar age which had not been subjected to separation of the components. Histochemical development of the recombinations was also remarkably normal. Neutral and acid mucopolysaccharide distribution in the enamel organ and dental papilla were as described *in vivo*. Glycogen distribution in the enamel organ was as *in vivo*, and, unlike the distribution in cultured fragments of the mandible as described in Part II - the glycogen distribution in the dental papilla was as *in vivo*, i.e. confined to the/

the periphery. In the cultured non-separated fragments of the mandible, glycogen tended to show a diffuse distribution throughout the dental papilla.

The rates of development of the recombinations were also comparable to those of the intact explants - 45% for the recombined explants from the 11-day stage mandibles, and 29% for those of the 12-day stage. The explants from the 11-day stage thus showed a higher rate of development, and also more normal morphological structure involving elongation of the enamel organ walls (see Figs. 79-82) than the explants from the 12-day stage. The former were oxygenated during culture, the latter were cultured in air.

The position of development in the explants, of the molar tooth germs which differentiated during culture, gives rise to speculation of the roles of the epithelial and mesenchymal components on inducing tooth formation. Due to the shrinkage of the epithelium in all cases, the masses of mesenchymal tissue forming the mandibles, were not completely covered by epithelium. Differentiation of the mesenchyme, however, showed what would appear to be their normal structures in normal anatomical relationship to each other, Meckel's cartilage represented as two rods of cartilage approaching each other anteriorly, and surrounded and replaced in part, by osteoid tissue as in vivo. Centrally in the explants, the mesenchyme had formed elevated tongues.

Detailed histological examination showed that tooth formation only took place where epithelium and mesenchyme were in contact in the anatomically normal position for tooth formation in relation to the mesenchymal structures. Where epithelium was not in contact with mesenchyme in such a position, no differentiation of the exposed mesenchyme was seen - and where epithelium was very close, but not at the normal position (see Fig. 81), then cyst formation of the epithelium there was common. No tooth germ was formed in an anatomically abnormal site in the mesenchyme.

Such observations lead to the speculation that the position and presence of the specific mesenchyme seems more important in the/

the induction and maintenance of the tooth germ than the epithelium. Those areas of epithelium, potentially essential for tooth formation, could of course have degenerated as a result of the separation procedures, but the presence of respiratory epithelium in the reconstituted layer, and development of salivary gland and tooth germs in their normal mesodermal anatomical positions in all the cultures that survived, would argue against this view. Salivary gland tissues developed in the separated and recombined tissues of both 11- and 12-day stage mandibles. These structures, known to be induced by the mesenchymal capsule (Borghese, 1950b; Grobstein, 1953b) were noted to have developed - like the tooth germs - in the anatomically correct position with respect to mesenchymal structure.

The present experiments were designed principally, however, not to investigate the role of epithelium and mesenchyme in inducing or maintaining odontogenesis, but to investigate the role of these tissues in determining future tooth morphology. With this in view, the recombinations of 11- and 12- day stage mandibular tissues were made, as already described, with incisor epithelium recombined with molar mesenchyme, and molar epithelium recombined with incisor mesenchyme.

The tooth germs developing from such recombinations were, once more, remarkably similar to those *in vivo* and those developing *in vitro* from similar stages of intact mandible.

The morphology of the tooth germ developing from the recombined transposed tissues of the 11-day old embryo, was the most equivocal, although most of the evidence points to it being incisiform, rather than molariform. One wall of the enamel organ appeared not to have elongated as expected, but all other histological details, including predentine formation at the incisal tip, were remarkably similar to those of a 17-day stage incisor tooth germ *in vivo*. The histochemical details were also compatible with such/

such a conclusion (Fig. 95). Its rate of development in culture was approximately 43% that of normal, a much higher rate than from the equivalent stage of intact mandible in culture.

The tooth germ was identified as having been formed from the combination of incisor epithelium, and molar mesenchyme. It would appear that in the 11-day old embryo - when the first morphological signs of odontogenesis are seen as the epithelial thickenings of the dental lamina - it is the epithelium which controls future tooth morphology.

Whereas the identification of the morphological features of the tooth germ developing from the recombined transposed tissues of the 11-day stage mandible make it possible to speculate only with some hesitation on the roles of the epithelium and mesenchyme in determining tooth form, the distinctive morphology of the tooth germs developing under similar circumstances, but from the 12-day stage, give more certain results.

At the 12-day stage, two tooth germs developed within one explant which was recombined after rotation of the mandibular epithelium. One tooth germ was incisiform, with a narrow incisal tip and elongation of the walls of the enamel organ where one side was more fully differentiated than the other, as in the incisor *in vivo*. The other tooth germ was molariform with a broad presumptive occlusal surface showing cusp formation, and short enamel organ walls relative to the width of the occlusal surface and cervical loop. Histochemical differentiation was very similar to that *in vivo* of 16-17 day stage tooth germs. The rates of development in culture were 35-45% that of normal.

The incisiform tooth germ developed from the combination of incisor epithelium and molar mesenchyme. The molariform tooth germ developed from the combination of molar epithelium and incisor mesenchyme.

These/

These results seem to indicate that at the 12-day stage, when the dental lamina is beginning to invaginate into the underlying mesenchyme, it is the epithelium which is dictating future tooth morphology.

Although the observations have established the role of odontogenic epithelium as controlling future tooth form at the 12-day, and possibly 11-day, stages in vivo, the epithelium clearly does not dictate all aspects of this form. Considered as entities, the tooth germs developing from the transposed recombined tissues were remarkably similar in morphological, histological and histochemical details to those in vivo. The relationships of the tooth germs to each other, and to other mandibular structures, however, were abnormal.

If the epithelial layer determines the orientation of the tooth within the mandible, then rotation of the epithelium on the mesenchyme through 180° should cause the incisor tip to appear dorsally in the mesenchyme and the cervical loop to elongate anteriorly towards the developing molar tooth germ. This, however, was not the case (see Fig. 89). The incisiform tooth germs in both 11- and 12-day stage recombinations, although transposed to the dorsal position in the mesenchyme as described, retained the orientation of the long axis of the tooth germ within the mesenchyme as in vivo. The incisal tip pointed anteriorly, and the elongation of the cervical loop advanced dorsally, i.e. away from the developing molar tooth germ.

If, after the fate of the cells had been determined, physical forces rotated the tooth germ about its dental lamina so that the cervical loop elongated dorsally in the mesenchyme rather than anteriorly, then the ameloblasts would have appeared on the lingual not the labial wall. Such was not the case. The labial wall, as in vivo, was more fully differentiated and resembled the potential site of enamel formation. Hence, although the ultimate nature of the/

the tooth germs seems to depend on the epithelium for its morphological form, it seems to be dependent on the mesenchyme for its axial orientation. Lack of advanced cusp differentiation in the molar tooth germ, prevents a similar analysis being made of its orientation within the mandible.

No single gradient of morphogenetic influence either within the epithelium or within the mesenchyme, as suggested by Butler (1967), would appear to be solely responsible for all aspects of the development of a heterodont dentition in mammals. Complex interactions between odontogenic epithelium and mesenchyme are obviously involved even from the earliest stages.

With the transposition of the incisor tooth germ to the dorsal region of the mesenchyme, it came to have an abnormal spatial relationship with Meckel's cartilage and its surrounding osteoid tissue. In vivo, the sheath of alveolar bone fails to cover the tip of the incisor tooth germ : however, under the transposed conditions, osteoid tissue extended anteriorly in culture to cover the tip (see Fig. 89, 90). Osteoid tissue appeared to be actually invading the labial wall and anterior tip of the enamel organ formed from recombination of tissues of the 12-day stage. Epithelium is known to induce surrounding bone in normal circumstances (e.g. around the developing eye, Coulombre, Coulombre & Mehta, 1962), and in pathological circumstances (e.g. in the urinary tract, Huggins, 1931), and the possibility that the tooth germ induces surrounding alveolar bone formation has already been discussed (Part II). However, the invasion of an epithelial structure by bone seems unusual. The most plausable explanation is that the invasion of the osteoid tissue has mimicked the invasion of blood capillaries into the enamel organ, normally found in the incisor tooth germ in vivo at a comparable stage of development, i.e. just before hard tissue deposition.

Having/

Having found that epithelium seems to dictate future tooth morphology at the 12-day stage, further experiments were planned to extend our knowledge of the epithelio-mesenchymal interactions by recombining separated tissues from this stage of development, with separated tissues from the 9-day stage, known to form only cuspless tooth germs on culture of the intact tissues (Part II).

Although the results were inconclusive, the experiments were remarkable for the survival of both components of the recombined explants in contrast with the homologous 9-day stage recombinations which invariably perished. Perhaps future experiments in improved conditions may be more meaningful.

Although no tooth germs developed from the recombinations of 9-day stage epithelium and 12-day stage mesenchyme, what was probably a rudimentary attempt at tooth formation was observed in the recombination of 12-day stage epithelium and 9-day stage mesenchyme. The structure consisted only of a cone of condensed mesenchymal cells surrounded by a large epithelial invagination into the mesenchyme.

If we assume from those appearances that tooth formation has at least been attempted, then we are encouraged to assemble some possible explanations for the results:

a) 12-day stage mesenchyme - known already to be capable of tooth formation - may be incapable at that advanced stage of stimulating 9-day stage epithelium into odontogenic activity.

b) 9-day stage epithelium may have been incompetent to react to the stimulus from the 12-day stage mesenchyme.

c) 9-day stage mesenchyme - was capable of inducing odontogenic activity in the competent 12-day stage epithelium. This epithelium, however, was incapable of cusp formation - either due to poor culture conditions, or lack of initial stimulus from /

from the 9-day stage mesenchyme.

d) 12-day stage epithelium may have induced the 9-day stage mesenchyme to be incorporated into the dental papilla of the tooth germ.

Although the 9-day and 12-day cross-recombinations gave little insight into epithelio-mesenchymal interactions in teeth, quite useful information emerged incidentally about the mechanism of salivary gland formation.

No salivary gland developed in the recombination of 9-day stage mesenchyme and 12-day stage epithelium, despite the differentiation of a healthy and extensive epithelial layer showing possible tooth formation. Salivary gland did develop in culture from recombinations of 9-day stage epithelium and 12-day stage mesenchyme. One gland showed the differentiation only of about three adenomeres, the other of about eight adenomeres.

Such results seem to confirm the theories of Borghese (1950b) and Grobstein (1953a, b, c). They found that branching of the epithelium of the submandibular salivary gland was dependent on the presence of the condensed mesenchymal capsule of the gland at the 12-day stage and older. They concluded that the mesenchyme contained a factor promoting this branching.

The results of experiments with the transposed tissues as described above are quite consistent with this belief : the mesenchyme of the 12-day stage mandible was capable of promoting branching of the 9-day stage epithelium : the less mature mesenchyme of the 9-day stage was incapable of promoting branching of the 12-day stage epithelium, in spite of the known invagination of the epithelium in the mandible to form the gland at that time.

From the results of all the preceding experiments with transposed recombined mandibular tissues, the most convincing conclusion concerning epithelio-mesenchymal interactions in tooth formation, is that the epithelium plays a dominant role in controlling future tooth morphology at the 12-day stage. This result/

result appears to conflict with the results of Kollar & Baird (1969) who found mesenchyme to be in control of tooth morphology. However, the conflict may be more apparent than real, for they studied interactions at later stages (13-16 days), and differences may merely lie in the complexity of reciprocal reactions at different stages in development.

The results of their experiments with the 13-day stage mouse embryonic tissues were found by them to be more equivocal than from older stages. However, difficulties in interpreting the different expressions of histodifferentiation and morpho-differentiation of the component tissues when isolated, recombined and cultured at these quite advanced stages of development, and the further complication of the inevitable distortion of the tissues in culture, leads me to believe that the importance of the epithelium and mesenchyme in the control of tooth form at these later stages is still open to question.

SUMMARY

Interactions between odontogenic epithelium and mesenchyme in mandibles of the EZL mouse at 8-12 days gestation age, were investigated.

The epithelium was found to be capable of clean separation as a layer from the underlying mesenchyme at all stages, by the action of trypsin-pancreatin in calcium-and-magnesium-free Tyrode's solution.

The viability of the tissues was tested by culture after recombination:-

a) 8-day stage tissues were capable of survival in culture, but appeared to lack the ability to form epithelial derviatives.

b) 9- and 10-day stage tissues were found difficult to manipulate and culture, and they rapidly degenerated.

c) 11- and 12-day stage tissues, reformed identifiable tooth germs.

Isolated epithelium and mesenchyme were incapable of tooth structure formation when cultured.

Results of experiments with epithelium and mesenchyme recombined as in vivo, or experimentally transposed, suggested that:-

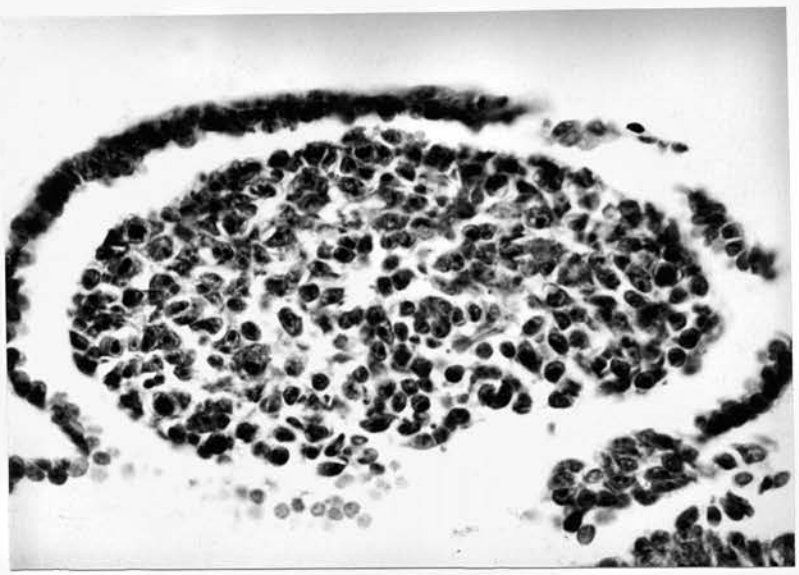
a) mesenchyme may be responsible for inducing and maintaining tooth germ formation in the mandible at early stages of odontogenesis,

b) the axis of development of the incisor within the mandible may be determined by the mesenchyme at the 11- and 12-day stages,

c)/

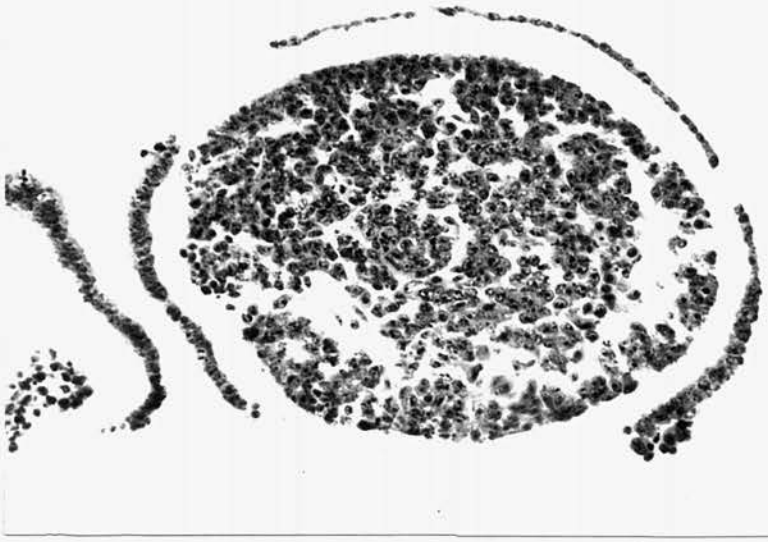
c) at the 12-day stage, and possibly at the 11-day stage, the odontogenic epithelium is responsible for dictating future tooth morphology.

FIGURES 70 - 95.



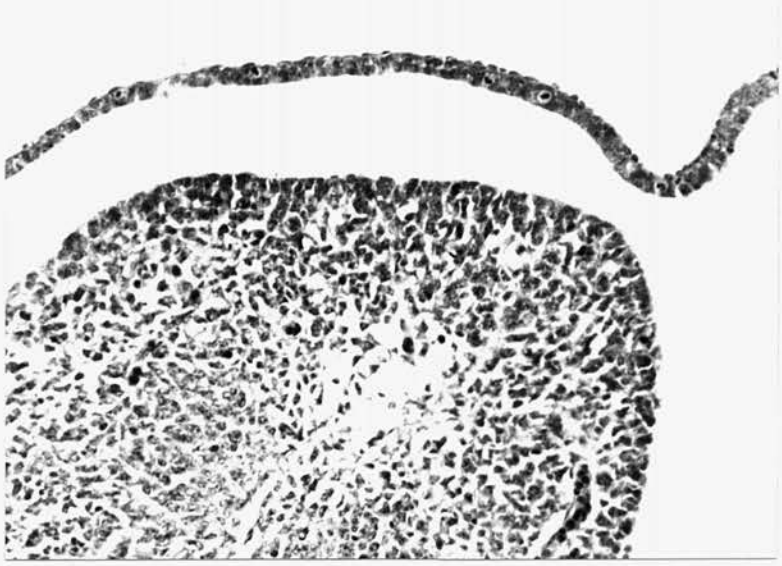
Allochrome xI00.

Fig. 70. 8 day embryo : Mandible.
Separated epithelium.



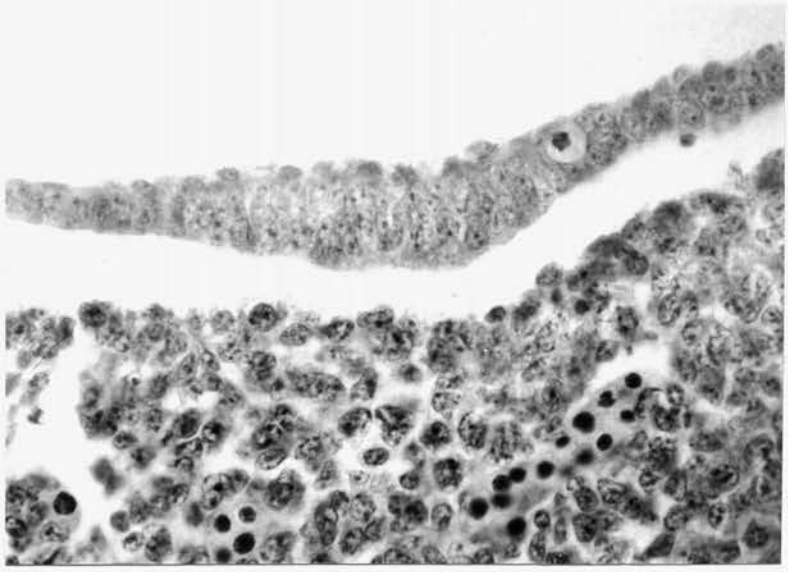
H. & E. x63.

Fig. 7I 9 day embryo : Mandible.
Separated epithelium.



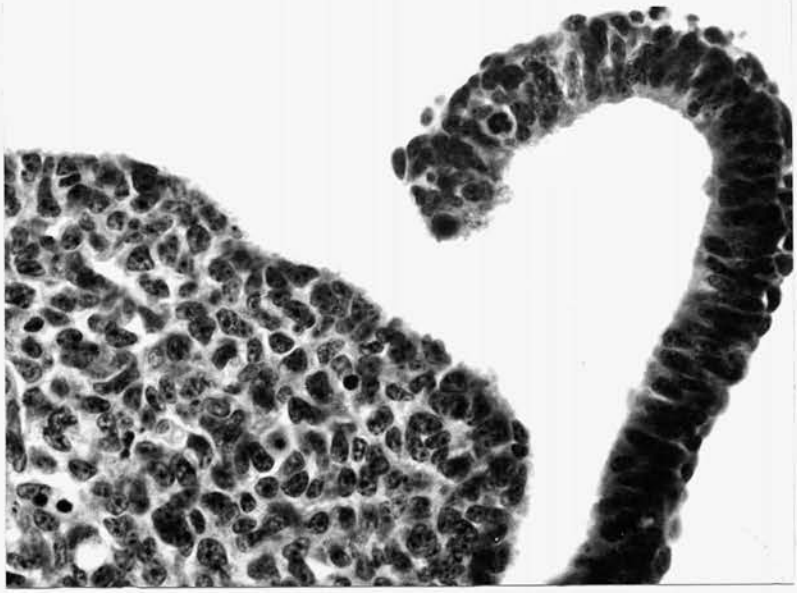
H. & E. x63.

Fig. 72. IO day embryo : Mandible. Separated epithelium.



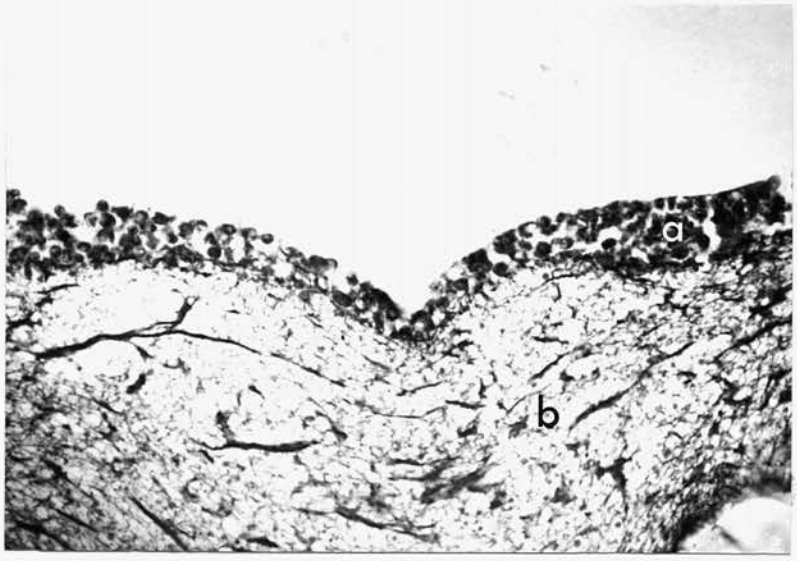
Haem. & Alcian blue xI60.

Fig. 73. II day embryo : Mandible. Separated dental lamina.



H. & E. xI60.

Fig. 74. I2 day embryo : Mandible. Separated epithelium

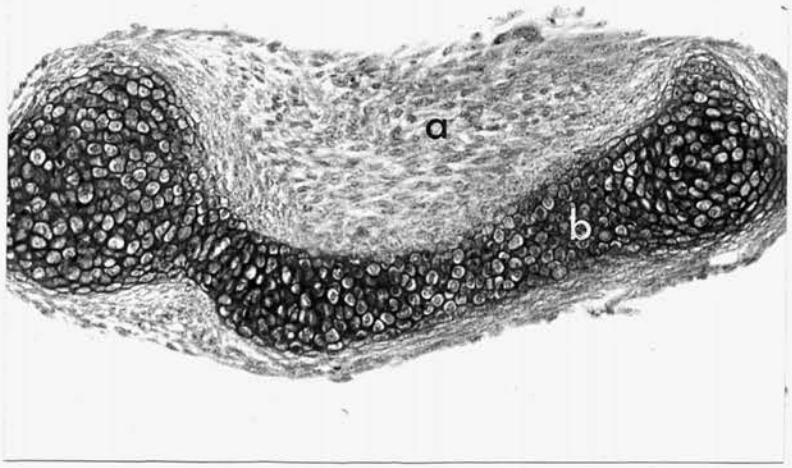


a) epithelium

b) collagen gel.

Allochrome xI00.

Fig. 75. Isolated epithelium on collagen gel - in vitro. I0 days culture.

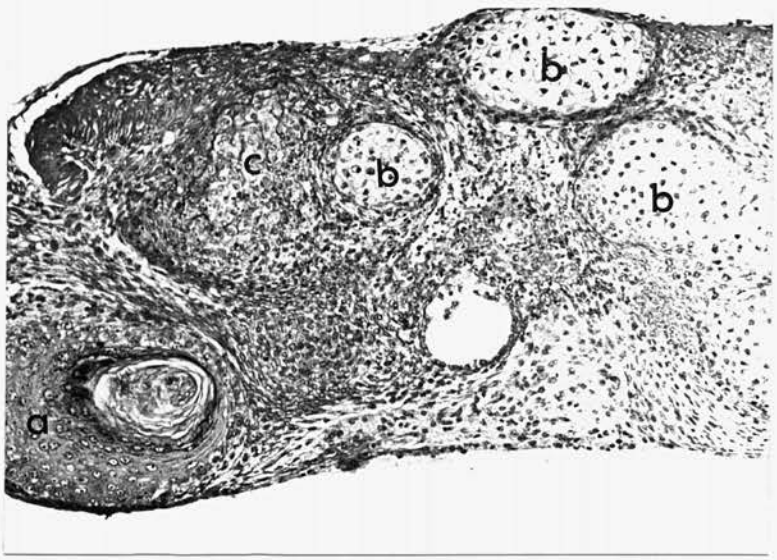


a) mesenchyme.

b) Meckel's cartilage.

Haem. & Alcian blue x63.

Fig. 76. Isolated mesenchyme in vitro.
IO days in culture.



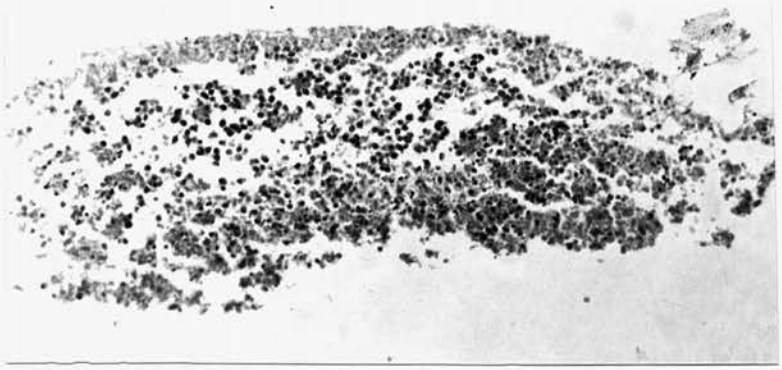
a) epithelium.

b) cartilage.

c) osteoid tissue.

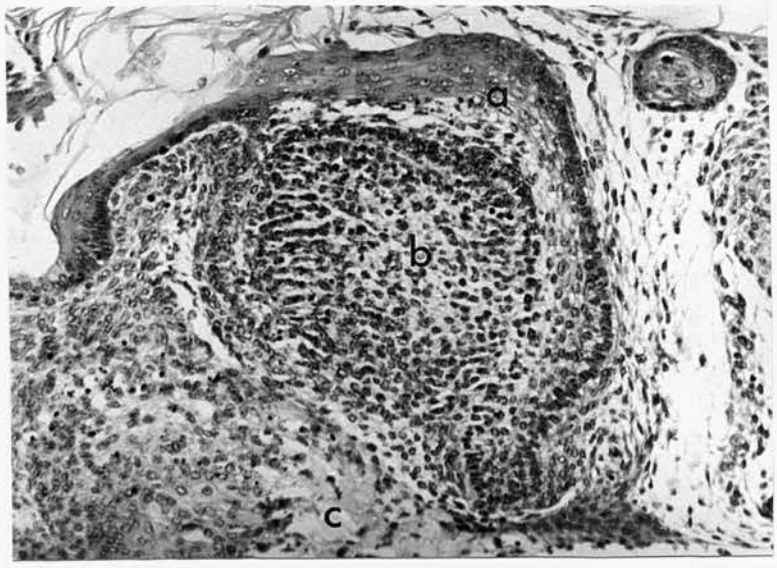
Allochrome & diastase x40.

Fig. 77. Mandible : recombined mesenchyme and epithelium.
8 day embryo.
I2 days in culture.



Haem. & PAS x50.

Fig. 78. Mandible : recombined epithelium and mesenchyme.
IO day embryo.
8 days in culture.



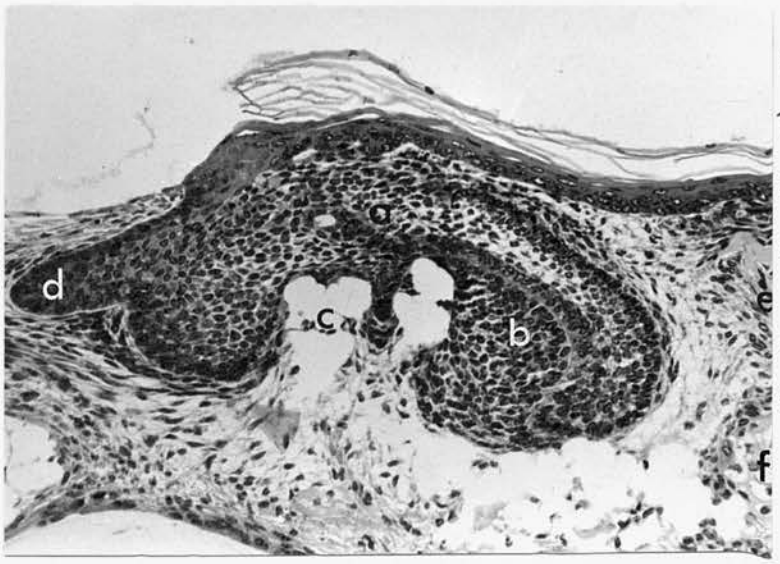
a) enamel organ.

b) dental papilla.

c) osteoid tissue.

H. & E. x63.

Fig. 79. Mandible : recombined epithelium and mesenchyme forming M_1.
II day embryo.
II days in culture.



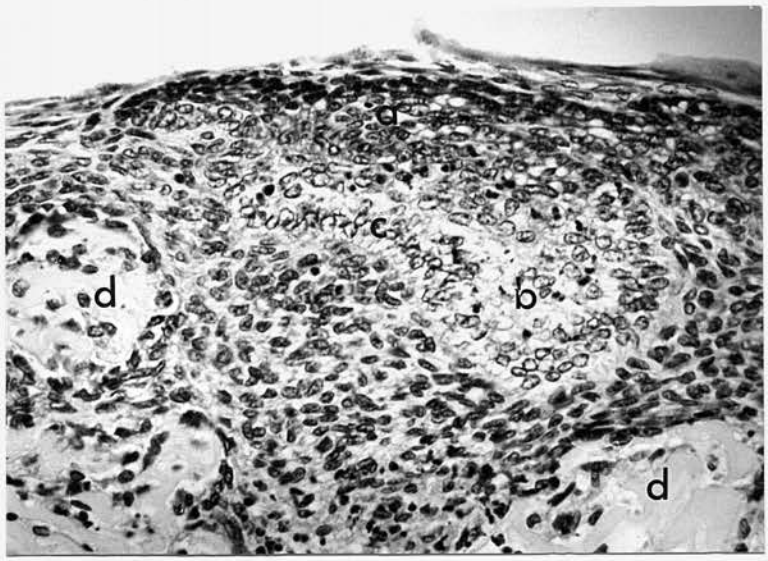
a) enamel organ.
b) dental papilla.
c) 'ghost' of raft.
d) dental lamina for M_2.
e) osteoid tissue.
f) cartilage.

H. & E. x63.

Fig. 80. Mandible : recombined epithelium and mesenchyme forming M_1.
II day embryo.
II days in culture

a) tongue.
b) dorsal end of tooth (of Fig. 80).
c) cyst.
r) 'ghost' of raft.

Fig. 81. Mandible - as in Fig. 80. x20
coronal section.
II day embryo.
To show tooth formation on one side of tongue : cyst formation on the other.



H. & E. xI00.

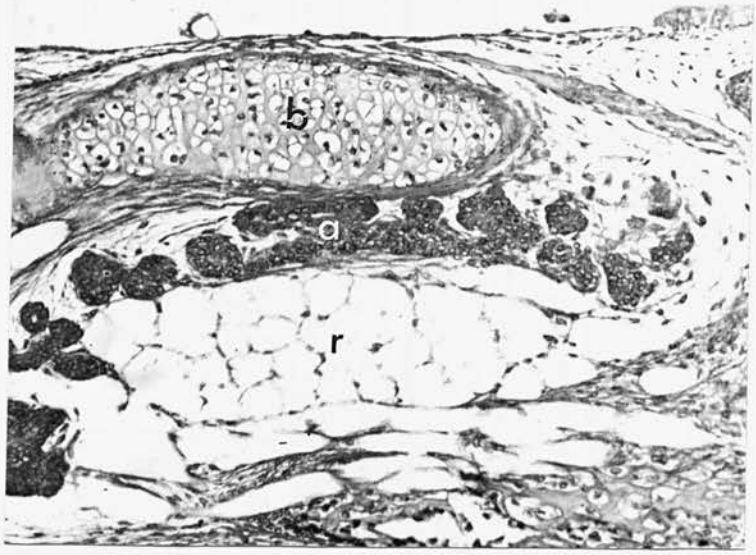
Fig. 82. Mandible : recombined epithelium and mesenchyme forming M_1.
I2 day embryo.
I4 days in culture.

a) enamel organ.

b) stellate reticulum.

c) internal enamel epithelium.

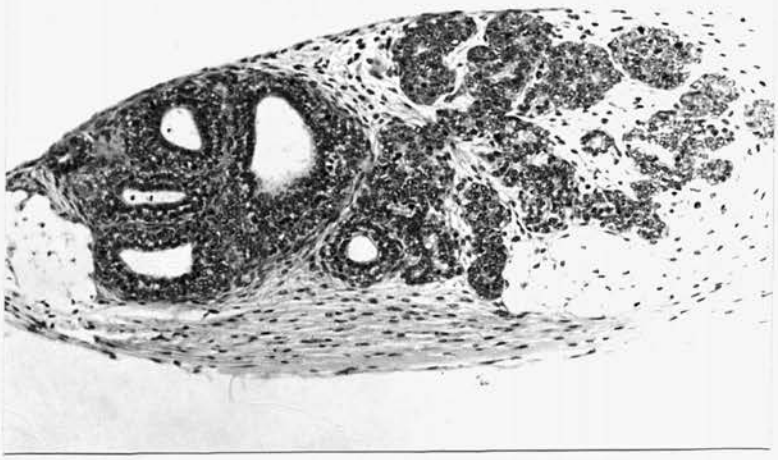
d) osteoid tissue.



a) salivary gland.
b) cartilage.
r) raft

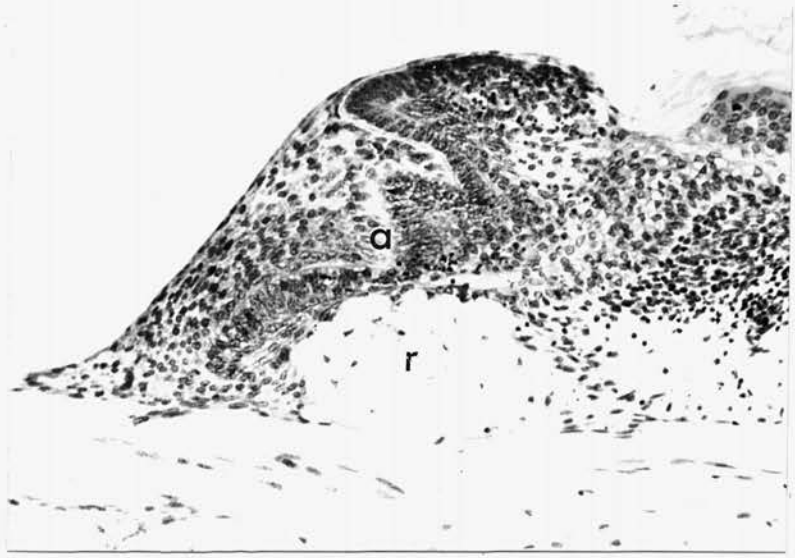
Allochrome x50.

Fig. 83. Mandible : recombined epithelium and mesenchyme forming salivary gland.
II day embryo.
II days in culture.



H. & E. x50.

Fig. 84. Mandible : recombined epithelium and mesenchyme forming salivary gland.
I2 day embryo.
I4 days in culture.

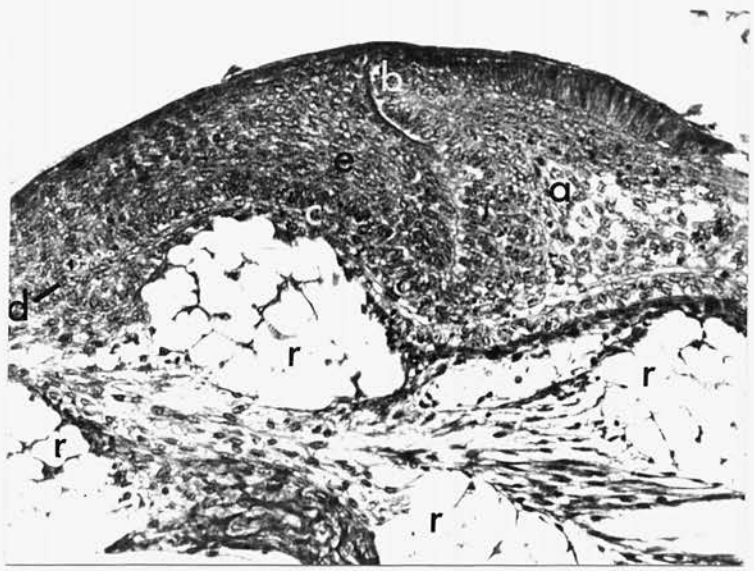


a) predentine.

r) raft.

H. & E. x63.

Fig. 85. Recombination of incisor epithelium and molar mesenchyme.
II day embryo.
I4 days in culture.
To show resemblance to molar.



a) stellate reticulum

b) short wall of enamel organ.

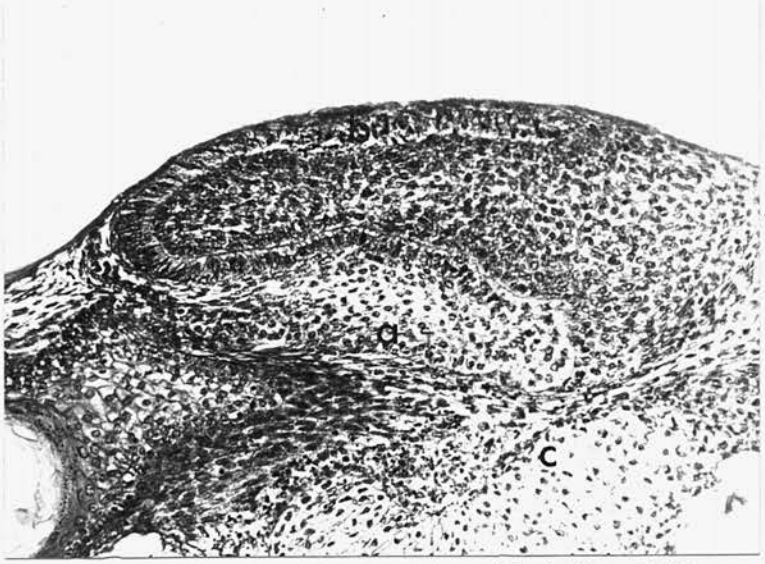
c) elongated wall of enamel organ.

d) cervical loop.

r) raft.

Allochrome & diastase x63.

Fig. 86. Same tooth germ as Fig. 85.
II day embryo.
To show resemblance to incisor.



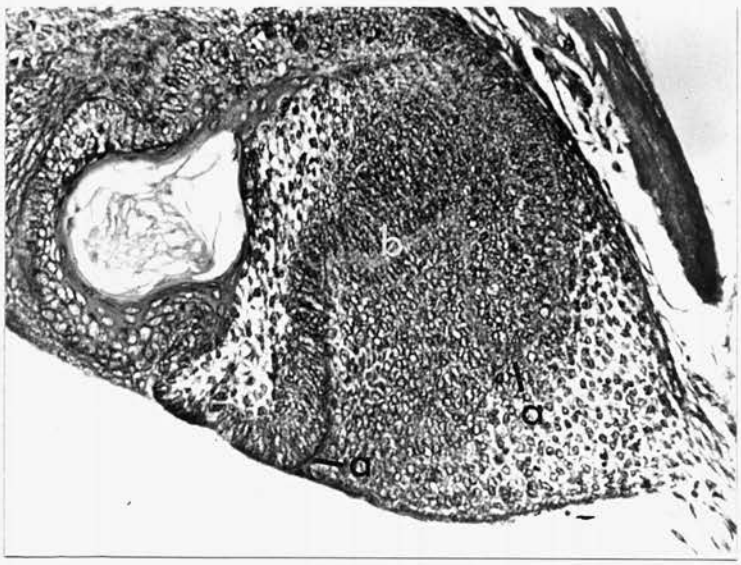
a) 'labial' wall of enamel organ.

b) 'lingual' wall of enamel organ.

c) cartilage.

H. & E. x63.

Fig. 87. Recombination of incisor epithelium and molar mesenchyme.
I2 day embryo.
II days in culture.

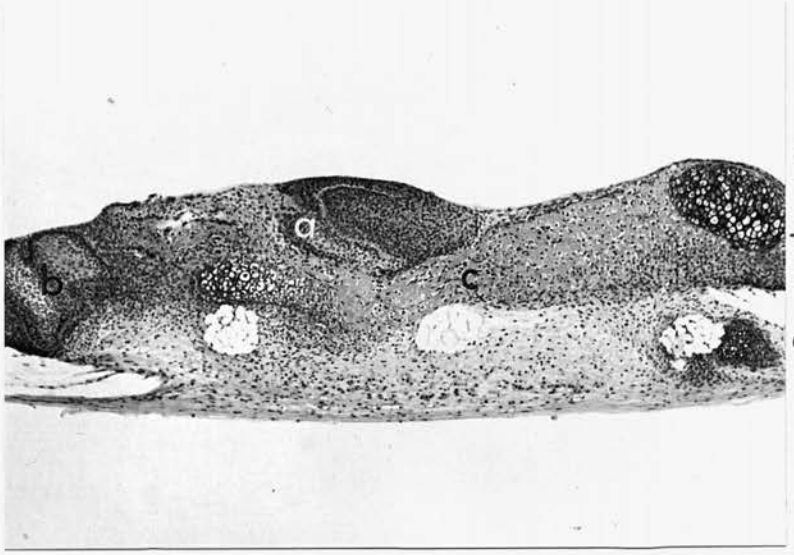


a) cervical loop.

b) early cusp formation.

Allochrome x63.

Fig. 88. Recombination of molar epithelium and incisor mesenchyme.
I2 day embryo.
II days in culture.



a) incisiform tooth germ.

b) molariform tooth germ.

c) Meckel's cartilage (+ endochondral) ossification.)

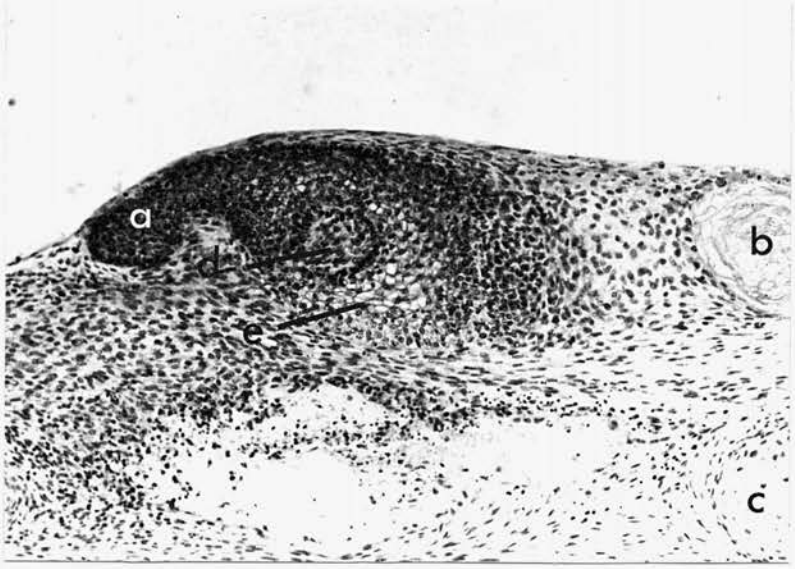
Alcian blue xI6.

Fig. 89. Mandible : recombined transposed epithelium and mesenchyme.
I2 day embryo.
Low power view of explant - tooth germs of Figs. 87 and 88.

a) invasion of osteoid tissue.

Alcian blue x63.

Fig. 90. Same tooth germ as Fig. 87.
(Incisor epithelium and molar mesenchyme).
I2 day embryo.
To show invasion of enamel organ by osteoid tissue.



a) epithelium.
b) epithelial pearl.
c) cartilage.
d) ? dental papilla.
e) ? stellate reticulum.

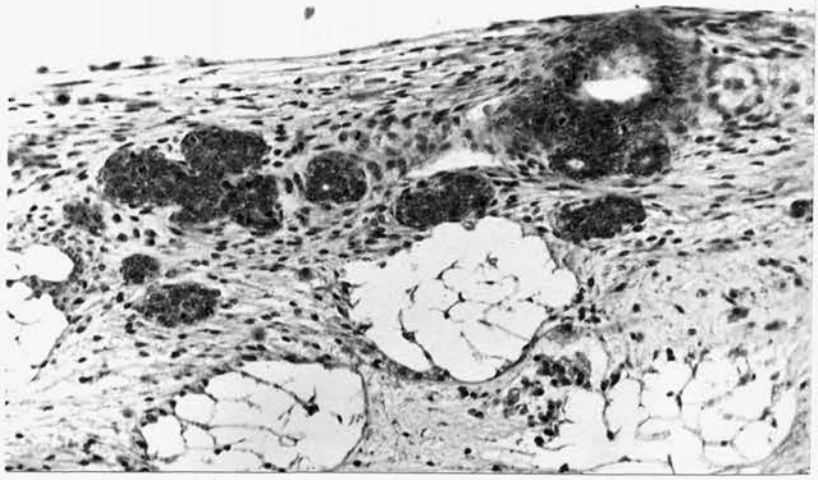
H. & E. x50.

Fig. 9I. Recombination of I2 day epithelium and 9 day mesenchyme.
I6 days in culture.
To show rudimentary attempt at tooth formation ?

a) salivary gland ducts.
r) raft.

Allochrome xI00.

Fig. 92. Recombination of 9 day epithelium and I2 day mesenchyme.
I6 days in culture.
Salivary gland duct formation.



H. & PAS x50.

Fig. 93. Recombination of 9 day epithelium and 12 day mesenchyme.
11 days in culture.
Salivary gland adenomere formation.

DEVELOPMENT OF ODONTOGENIC EPITHELIUM AND MESENCHYME IN VITRO :- TISSUES SEPARATED AND RECOMBINED

INCISOR EPITHELIUM COMBINED WITH INCISOR MESENCHYME
MOLAR EPITHELIUM COMBINED WITH MOLAR MESENCHYME

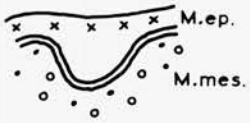
AGE AT EXPL'TN	APPEARANCE OF TISSUES AT EXPLANTATION	APPEARANCE OF TISSUES AFTER CULTIVATION	APPEARANCE OF NEAREST EQUIVALENT IN VIVO STAGE
11D		A.  11D CULTURE	 16D
		B.  11D CULTURE	 16D
12D		 14D CULTURE	 15D HISTOLOGICAL EQUIVALENT  16D HISTOCHEMICAL EQUIVALENT

Fig. 94.

x : glycogen.

o : neutral mucopolysaccharide.

● : acid mucopolysaccharide.

DEVELOPMENT OF ODONTOGENIC EPITHELIUM AND MESENCHYME IN VITRO :- TISSUES SEPARATED AND RECOMBINED

INCISOR EPITHELIUM COMBINED WITH MOLAR MESENCHYME
MOLAR EPITHELIUM COMBINED WITH INCISOR MESENCHYME

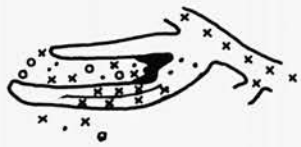
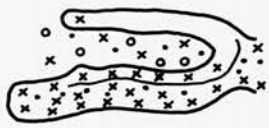
AGE AT EXPL'TN	APPEARANCE OF TISSUES AT EXPLANTATION	APPEARANCE OF TISSUES AFTER CULTIVATION	APPEARANCE OF NEAREST EQUIVALENT IN VIVO STAGE
11D		 14D CULTURE (No available data on • in stellate reticulum)	 17D
12D A.		 11D CULTURE	 16D
B.		 11D CULTURE	 17D

Fig. 95.

x : glycogen.

o : neutral mucopolysaccharide.

• : acid mucopolysaccharide.

GENERAL DISCUSSION.

GENERAL DISCUSSION

Tooth germs are not alone among developing organs in showing, as their first signs of morphological differentiation, a localised thickening of the epithelial layer, and an underlying condensation of the mesenchymal cells. Such a pattern is common in epidermal derivatives - for example, in hair follicles, vibrissae, feathers, pancreas, salivary gland, lung and the developing limb bud.

Further development of these derivatives, leading to structures with widely differing appearances and functions, seems to involve as many different patterns of induction as there are organs. Out of the numerous reports on their development, however, some general patterns seem to be emerging, and it is instructive to compare and contrast the pattern of tooth development as we have found it, with patterns known to exist for other organs.

The very early determination of mouse tissues for tooth formation, agrees well with the theory of Weiss (1939) and Waddington (1962), that tissues are determined and show self-differentiation in culture before histodifferentiation takes place: the ability to differentiate in culture into cuspless teeth is acquired two days before the dental lamina makes its first appearance, and the ability to differentiate into teeth of different morphology, is acquired one day before it appears. Despite the widely held view that most - if not all - organs possess the same property, few actual reports can be found in the literature of such examples. Certainly, however, it has been shown that limb buds (Saunders & Gasseling, 1968) and the pancreatic gland (Wessells & Cohen, 1967; Wessells, 1968) are capable of self-differentiation in culture from a stage where only undifferentiated tissues are seen on light microscopic examination.

Similarly/

Similarly to the developing tooth germ, these organs also acquire their specificity in a step-wise manner. The limb bud is found to become determined first for the antero-posterior axis, and later for the proximo-distal axis (Saunders & Gasseling, 1968); isolation and culture of presumptive pancreatic tissue shows a stage where no determination can be demonstrated, followed by two recognisably different stages. In the first stage, the epithelium is dependent on its own specific mesenchyme for its development, but in the second stage, it differentiates into pancreatic tissue when maintained experimentally by any mesenchyme so far tested (Golosow & Crobstein, 1962; Wessells, 1968).

Interactions between the individual tissues from which organs are formed have recently been reviewed by Grobstein (1967). He concluded that where organ rudiments form from two or more tissues, these tissues show reciprocal dependence on each other for their development.

In general, mesenchyme has been found to show principally two types of activity when involved in the development of epidermal derivatives. Firstly, in most organs it stimulates the overlying epithelium into its apparent initial activity. Such examples are found in developing limb buds (Saunders & Gasseling, 1968); feathers (Sengel, 1956; Rawles, 1963; Wessells, 1965); thymus (Auerbach, 1960); salivary gland (Borghese, 1950b; Grobstein, 1953a); lung (Dameron, 1961; Alescio & Cassini, 1962); liver (Le Douarin, 1963, 1964a, b); preen gland (Gomot, 1958) and pancreas (Wessells, 1968). Organogenesis does progress where it seems to be the epithelium which stimulates initial activity in the mesenchyme - the ureteric bud, for example, is thought to stimulate the underlying mesenchyme into nephrogenic differentiation (Grobstein, 1955). In many cases (e.g. Sengel, 1956; Saunders & Gasseling, 1968), the initial inductive activity of the mesoderm is short-lived, and the definition of the 'initial' stimulus may be hard/

hard to prove. Apparent differences in pattern of development between organs may be due solely to comparison of different stages of differentiation.

Secondly, in contrast to the short term inductive stimulus, the mesenchyme seems to exert a maintenance effect on the overlying epithelium over a prolonged period of time. This effect is capable of influencing, experimentally, the epithelial differentiation towards the type usually in contact with the particular mesenchyme (McLoughlin, 1960a, b; 1961b, 1968; Billingham & Silvers, 1968). The presence of both mesenchymal effects has been clearly shown in the developing limb bud. Not only was mesenchyme proved to induce the formation of the apical ectodermal ridge, but its continued presence was found necessary to maintain the ridge (Zwilling, 1956; Zwilling & Hansborough, 1956).

A search for similar activities of the mesenchyme in the present experiments in early tooth development has failed due to the difficulties of separating and recombining explants from young embryos capable of survival *in vitro*, and from a lack of experiments recombining heterologous and odontogenic tissues. However, as was discussed in Part III, the results of experiments where odontogenic tissues were separated from 11- and 12-day mouse embryos, and recombined again as *in vivo*, suggest that it is the specific mesenchyme which determines the presence of any developing tooth germ. Influence of the mesenchyme on the epithelium also seems to account for the orientation of the axis of development of the incisor tooth germ within the mandible. In this respect, the tooth germ shows a pattern of early development similar to that of the preen gland. On rotation, the uropygial epithelial invagination was found to follow the orientation of the mesenchyme (Gomot, 1958).

As for the part played by the epithelium in the reciprocal reactions in organogenesis, much confusion seems to exist in the reports/

reports. Although it is certain that the part played by the epithelium in differentiation depends upon the particular organ, some confusion possibly arises due to attempts to compare tissues from various organs at incomparable stages in their development and stability of determination.

Billingham & Silvers (1968) believe that most of the experimental findings suggest that no predetermined regional specificity is present in the early embryonic ectoderm, and that its qualitative regional differentiation is initiated by stimuli from the subjacent mesenchyme. More credible, however, is the assumption of Grobstein (1968) that the epithelium, although dependent on the mesenchyme for its initial activity and continued development, is already covertly differentiated, in that its response to the stimulus from the mesenchyme is specific to itself. He quotes, for example, that although salivary gland epithelium and mesenchyme separated and recombined form salivary gland as expected, pancreatic epithelium, stimulated into activity by similar salivary gland mesenchyme, differentiates into pancreatic tissue. Waddington too (1962), believes that, with a few exceptions, the character of an induced organ is determined by the competence of the reacting tissue, and not by the nature of the inducer.

Examples of each type can be found in the literature. Prospective feather dermis is able to induce feather formation in epithelium destined under normal conditions to form surface epidermis or scale (Sengel, 1958; Rawles, 1963; Wessells, 1965, 1968). Hilfer (1968) interpreted the dependence of salivary gland epithelium, ureteric bud and lung epithelium on their own (isologous) mesenchyme for the expression of their typical organogenesis, as being further examples of the specificity of organ type being dependent on the mesenchyme. Zwilling (1955) found that the type of limb depended on the mesenchyme, although outgrowth depended on the presence of the epithelium.

On/

On the other hand, further evidence shows that epithelium certainly plays an active part in organogenesis in many cases. whereas Sengel, Rawles and Wessells, as quoted, found that feather dermis could induce feather formation in heterologous epithelium, Wang(1943) found that the details of feather structure were determined not by the dermal core which induced it, but by the place of origin of the follicular epidermis. Wessells(1968) found that in some cases, although the mesoderm named the type of organ , the epithelium determined the detailed structure. Thus anuran belly skin grafted over urodele mouth mesoderm, forms an anuran mouth. Taderera (1969, in press), found that mouse lung epithelium could be stimulated into activity by chicken lung mesoderm, but the pattern of branching was mammalian, rather than avian.

It is to this latter pattern of activity, that tooth germ development seems to belong. At the primitive tooth band stage, i.e. 11-12 days in the EZL strain of mouse, although it seems likely that the presence of the tooth germ and its axis of development are controlled by the mesenchyme, it appears to be the epithelium which dictates the future detailed morphology of the tooth.

Whether the odontogenic epithelium at this stage exhibits an active inductive stimulus on the underlying mesenchyme, controlling its morphological shape, or not, is unknown. Grobstein (1962) found that once epithelium had been exposed to an inductive stimulus for a sufficient length of time, it was capable of differentiation without the specific inducer. It has also been shown that prospective feather epidermis, once stabilized, can cause non - feather dermis to take part in feather formation (Sengel, 1958), and that scale epidermis can cause chick gizzard mesoderm to participate in forming scales (Wessells, 1968). It may be that the epithelium of/

of the tooth germ, by the time the dental lamina is formed, is already stabilized and its immediate action on the mesenchyme is only one of recruitment of the cells into the requisite pattern.

Whether mesenchyme does induce tooth formation and determine the axis of orientation within the mandible, and whether the epithelium is responsible for determining future tooth morphology, must be confirmed by more numerous and more varied experiments with all early stages of organogenesis of the tooth germ.

ACKNOWLEDGEMENTS

This work was carried out with the financial aid of the Medical Research Council, and under the supervision of Professor M. Swann, F.R.S., for whose continued interest I am very grateful.

The experimental work was carried out initially at the Department of Zoology, University of Edinburgh, and latterly at the Department of Physiology, University of Cambridge. I am indebted to the technical staffs of both Departments for help, and also to many colleagues for advice and criticism. In particular, I am deeply grateful to Dame Honor B. Fell, F.R.S., who granted me facilities at the Strangeways Research Laboratory, Cambridge, for learning the techniques of tissue culture, and from whose helpful discussions the final plan of the experiments evolved. My gratitude is also warmly extended to Professor E.N. Willmer, F.R.S., who made me feel so welcome in his Department, and who took so much interest in my work.

Secretarial help from Miss Mhairi MacKenzie in the preparation of the thesis has been of valuable assistance. Aid in the preparation of drawings and photographs for the thesis was ably rendered by the technical staff of the School of Dental Surgery, University of Edinburgh, under the supervision of Mr. A. Hunter, M.B.E., whose help and consideration has been outstanding. My thanks are also due to my colleagues at the School for discussions, and to Professor Boyes, F.D.S. for his continued help and co-operation.

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